"QUANTIFICATION OF MICROVESSEL DENSITY IN BREAST CARCINOMAS BASED ON IMMUNOHISTOCHEMISTRY"

DISSERTATION SUBMITTED FOR

M.D. DEGREE EXAMINATION

BRANCH III PATHOLOGY

OF

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY

CHENNAI

TIRUNELVELI MEDICAL COLLEGE HOSPITAL

TIRUNELVELI

APRIL -2015

CERTIFICATE

This is to certify that the Dissertation **"QUANTIFICATION OF MICROVESSEL DENSITY IN BREAST CARCINOMAS BASED ON IMMUNOHISTOCHEMISTRY"**presented herein by **Dr.SARANGAN. A** is an original work done in the Department of Pathology, Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D. (Branch III) Pathology under my guidance and supervision during the academic period of 2012 - 2015.

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I hereby certify that this work embodied in the dissertation **"QUANTIFICATION OF MICROVESSEL DENSITY IN BREAST CARCINOMAS BASED ON IMMUNOHISTOCHEMISTRY"** is a record of work done by **Dr. SARANGAN A** in the Department of Pathology, Tirunelveli Medical College, Tirunelveli, during his postgraduate degree course in the academic period 2012-2015. This work has not formed the basis for any previous award of any degree.

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DECLARATION

I solemnly declare that the dissertation titled **"QUANTIFICATION OF MICROVESSEL DENSITY IN BREAST CARCINOMAS BASED ON IMMUNOHISTOCHEMISTRY"** is done by me at Tirunelveli Medical College Hospital, Tirunelveli.

The dissertation is submitted to The Tamilnadu Dr. M.G.R. Medical University towards the partial fulfilment of requirements for the award of M.D. Degree (Branch III) in Pathology.

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ACKNOWLEDGEMENT

I take immense pleasure to acknowledge all those who have helped me to make this dissertation possible.

I am grateful to the **Dean, Tirunelveli Medical College** and **Medical Superintendent of the Tirunelveli Medical College Hospital** for permitting me to undertake this study.

I express my profound sense of gratitude to **Dr. K. Shantaraman,M.D.,** my respected Professor and Head of Department of Pathology, Tirunelveli Medical College, Tirunelveli and my guide **Dr.S.Vallimanalan, M.D.,** for his unstinted guidance and motivation.

I thank **Dr.K.Swaminathan, M.D., Dr. J. Suresh Durai, M.D., Dr. Arasi Rajesh, M.D.,** Professors of Pathology for their constant support and encouragement. I profusely thank all the other faculties and my postgraduate colleagues for their valuable support.

I sincerely thank the Professors and faculties of the Departments of General Surgery and Oncology for providing me the specimens for my study.

I also sincerely thank the Technicians and other members of the Department of Pathology and the Central Diagnostic Laboratory for their kind co-operation.

I thank all my family members for their encouragement and support during this study.

ABBREVIATIONS

DNA	: Deoxy ribonucleic acid
DPX	: Di-N-Butyle Phthalate in Xylene.
H&E	: Hematoxylin and Eosin
NOS	: Not Otherwise Specified
NST	: No Special Type
RBC	: Red Blood Corpuscle
SBR	: Scarff-Bloom-Richardson
S.D.	: Standard Deviation
sig	: Significant
SPF	: S Phase Fraction
TDLU	: Terminal Duct Lobular Unit
IHC	: Immunohistochemistry.
MVD	: Microvessel density

CONTENTS

QUANTIFICATION OF MICROVESSEL DENSITY IN BREAST CARCINOMAS BASED ON IMMUNOHISTOCHEMISTRY”

Abstract:

Breast cancer is the most frequent neoplasm causing death in women between 35-55 years of age. Angiogenesis is of key importance in the process of tumor progression in a number of tumor types including breast cancer. This is a both retrospective and prospective study which included fifty breast cancer cases . Angiogenesis was estimated by determining micro vessel counting after immune staining the paraffin embedded tissue sections using anti-cd34 antibody. The study is designed to compare MVD in different tumor stage, grade and age group. Age of the patients ranged from 35 to 85 years with a mean age of 52.58 years. Majority showed (40%) more than 84 vessels count in 1 mm^2 field area . Micro vascular density positively correlated lymph nodes involved by the tumor and grade of the tumour . In the future, antibodies specific to proliferating endothelium, together with the development of automated image analysis may improve the accuracy and value of measuring angiogenesis-induced microvessel density**.**

Key words: Breast Carcinoma, MVD, Cd- 34, Angiogenesis.

INTRODUCTION

Breast cancer is one of the most common carcinoma among women in India, which accounted for 25 to 30 percent of all cancer in woman[(1)]. Breast cancer is the second most common cancer with an estimated 115,251 new diagnoses[(2)] and the second most common cause of cancer related deaths with 53,592 breast cancer deaths in 2008[(2)]**.** The incidence of breast cancer increases with age. The incidence of female breast cancer is increasing between the age group of 35 to 50 years worldwide[3.] Invasive ductal carcinoma comprises the largest group of malignancy which constitutes about 65% - 80% of all breast carcinomas[4.]

Though there is an increase in breast cancer incidence, breast cancer mortality is decreasing in the last 20 years [(4).] This is mainly caused by both the introduction of breast cancer screening and use of adjuvant systemic chemotherapy. Breast are made up of specialized epithelium and stroma which may give rise to both benign and malignant neoplasm.

Patients who are at high risk can be identified based on clinical and pathological prognostic factors, such as age, menopausal status, size of the tumour, grade of the tumour, lymph node status and hormonal receptor status.

In addition to this there are certain predictive factors like,

1. Estrogen receptor and progesterone receptor.
2. HER2/neu amplification :

   HER2 /neu over expression is associated with poor prognosis. But the drug transtuzumab can be targeted against this oncoprotein.
3. Proliferative markers like Ki-67
4. Angiogenesis

One of the emerging predictive and prognostic factor in breast carcinoma is tumour induced angiogenesis. Angiogenes is defined as process of formation of new blood vessels from the endothelium of the already pre-existing vasculature**.**

It is important in growth, progression and spread of the tumour. So, tumour microvessels are necessary for sufficient supply of nutrients and oxygen and removal of waste metabolites from tumour cells.

In normal life, angiogenesis play a vital role in reproduction, embryogenesis, menstruation, and wound healing and repair. Its importance in solid tumours was first recognized by Folkman et al in 1971[(5)], he suggested that the growth of tumours was depends on angiogenesis. Micro vascular density is considered to be a hallmark of the angiogenesis.

The first study to examine intratumoral microvesseldensity (IMD) was carried out by Weidner and colleagues in 1991with immunohistochemistry. They used factor Vlll antigen as an endothelial marker in a series of breast

cancers [5]. Since this initial work many antibodies including those against CD31 & CD34has been used to assess MVD[6].

It has been emphasized that high micro vessels density in growing tumors closely associated with increasing number of tumor cells fell into the blood stream. In recent years, it has been proposed that quantification of intratumoral microvessel density by immunostaining for endothelial cell markers, such as CD34, CD31, von Willebrand factor, may be a useful prognostic factor in breast carcinomas.

So our present study was undertaken to asses and correlate the microvessel density in breast carcinomas with different clinicopathological factors and to ascertain the correlation of tumor angiogenesis with metastasis.

Aim of the study

AIMS AND OBJECTIVES

1. To quantify and grade the microvessel density in breast carcinomas with immunohistochemistry.
2. To asses and correlate the microvessel density in breast carcinomas with different clinicopathological entities with immunohistochemistry.
3. To ascertain the correlation of tumor angiogenesis with metastasis.

Review of Literature

REVIEW OF LITERATURE

HISTORY

Breast cancer was known but uncommon until the 19th century, in the year 1882 the first mastectomy for breast carcinoma was done by William stewart halsted. [9]. The procedure involved excising both breasts, lymph nodes, and the pectoralis muscles. Thereafter, the arrival of the Halsted radical mastectomy increased the survival rate by 50%.

Breast cancer staging systems was introduced in the 1920s and 1930s.[10]In 1926, first case-controlled study on breast cancer epidemiology was done by Janet Lane-Claypon [11],who was analysed the first epidemiology study on fertility and breast cancer risk.

The results from the Nurses Health Study (1995) and the reports of the Women's Health Initiative trial (2002) showed that the hormone replacement therapy could increase the incidence of breast cancer[12].

In 1945, Algire and Chalkley [14] were the first to determined that tumor growth is very closely related to the formation of an new microvessels.

In 1991, the first study to estimate the intratumoral microvessel density immunohistochemically was performed by Weidner and colleagues [13]**.** Theyused factor Vlll related antigen as an endothelial marker in a group of breast carcinomas.

ANATOMY

DEVELOPMENT OF BREAST:

The mammary glands develops from the ectodermal mammary ridges at the 5th week of intrauterine life. Bilaterally, they appears as a thickened line and it extends on the ventral surface of the fetus from the axillary to mid thigh. Around 7th week in utero major part of mammary ridge disappear**.**But some portion of it persist in the 4th or 5th intercostals space called the primary mammary buds**.**

Primary buds of ectoderm starts invading into the underlying developing stroma**.**By 10- 12th week of gestation, the primary mammary buds branches to form secondary buds, subsequently it forms the mammary lobules.

In 5th month of fetal development, the ectodermal penetration produces 15 –20 radial ingrowths branching into the developing breast**.** Small lumen will develop within the mammary buds which latter forms the developing lactiferous ducts and the branches of lactiferous duct**.**

The lactiferous ducts converge to open into a mammary pit, which then converted into nipple during infancy. There is no discernible variation between the male and female breast tissues from the time of conception until puberty.

While at puberty females exhibit branching and further lengthening of their ducts accompanied by lobular development and proliferation of fibrous

stroma and fat tissues reaching their maximum breast development by the age of 20 years.

The menstrual cycle is accompanied by minor variations of the breast tissue, but major physiological changes of the breast tissue are seen during pregnancy and lactation. There is major regression of the breast tissue during menopause which merges with aging associated atrophy of the breast.

The breast is a modified sweat gland covered by skin and subcutaneous tissue and lies over the pectoralis muscle, being separated from it by a fascia.

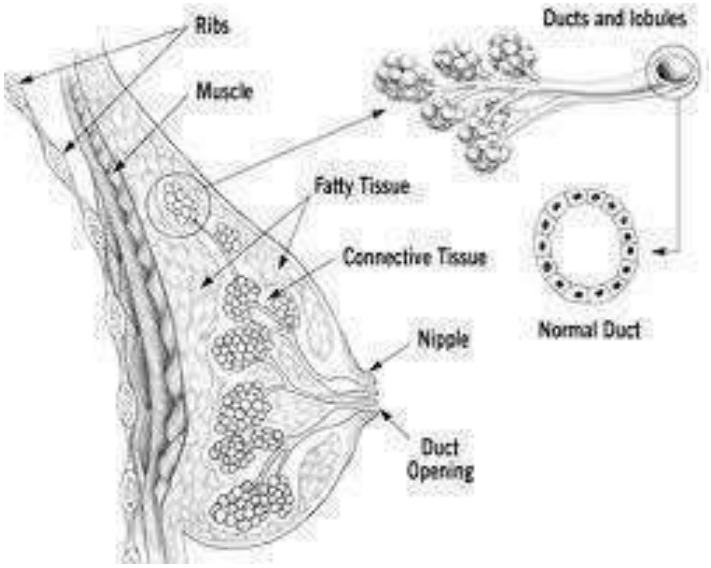

Fig 1 : Anatomy Of Breast

The functional unit of the organ is the glandular structure arranged into lobes which is made up of the following two components:

1. The terminal duct–lobular unit
2. The large duct system.

The terminal duct- lobular unit is represents the secretory portion of the gland which is composed of lobule and the terminal ductule**.**The breast development depends on the close interaction between these specialized epithelial and mesenchymal tissues. The large ducts contains only minimal amount of specialized stroma**.** The intralobular stroma envelopes the acini of the lobules. It consists of hormone responsive fibroblast-like cells and scattered lymphocytes. The stroma appears myxoid. The interlobular stroma is made of dense fibrous connective tissue admixed with adipose tissue.

BLOOD SUPPLY:

Breast is supplied by 3 major arteries and their branches:

- Predominant blood supplied by Internal thoracic artery, which is a branch from internal thoracic artery.
- Branches of the lateral thoracic, superior thoracic and acromiothoracic arteries.
- Lateral branches of posterior intercostal arteries.

Venous drainage of the breast follows the course of arteries forming an anastomotic circle in the subcutaneous tissue beneath the nipple-areolar complex. From this the veins run as

1. Superficial veins draining into internal thoracic vein.
2. Deep vein draining into internal thoracic, axillary and posterior intercostal veins.

LYMPHATIC DRAINAGE:

1. Axillary lymph nodes: Lymphatic drainage is mainly into the anterior group of axillary nodes**.** Posterior, lateral, central and apical groups of nodes also receive either directly or indirectly. These important nodes are arbitrarily divided into five groups**.**
    a. The lateral nodes lie posterior to the axillary vein and receive the lymph from the upper limb.
    b. The pectoral nodes located at the inferior border of the pectoralis minor, drain most of the breast.
    c. The subscapular nodes situated in the posterior axillary fold, drain the posterior shoulder.
    d. The central nodes lie near the base of the axilla, drain the lymph from the above three groups.
    e. The apical nodes lie medial to the axillary vein and superior to the pectoralis minor.The apical nodes receive the lymph from all the other groups**.**
2. The internal mammary nodes which lies along internal thoracic vessels.
3. Supraclavicular node, cephalic node, posterior intercostal, subdiaphragmatic and subperitoneal lymph plexus Most of the lymphatic vessels from the breast, upperlimb and the lymphatic vessels above the umbilicus drain into the axillary nodes.

Lymphatic vessels of breast:

1. The superficial lymphatics overlying skin of breast except nipple and areola**.** They pass radially to the surrounding lymph nodes (axillary, internal mammary,supraclavicular and cephalic node)
2. The deep lymphatics drain the parenchyma, nipple and areola of breast.

About 75% of lymph drains into axillary nodes, 20% into internal mammary nodes and 5% into posterior intercoastal nodes

NERVE SUPPLY:

Nerve supply is by anterior and lateral cutaneous branches of 4^{th} and 6^{th} intercostal nerves.

HISTOLOGY:

The keratinizing squamous epithelium of the overlying skin invaginates into the orifices of the nipple, then it is converted in to a double layered cuboidal epithelium**.**The entire ductal-lobular unit is lined by two cell layers, luminal epithelial cells and surrounded by layer of myoepithelial cells. Luminal cells will be columnar or cuboidal depending on their function..The entire glandular epithelial system lies on a continuous basement membrane. Occasional scattered endocrine cells are also found in normal breast. Both the luminal and myoepithelial cells thought to be arise from committed stem cells in the terminal duct.

The nipple area was formed by the lactiferous duct along with the sebaceous unit. The epidermis of nipple and areola is similar to that of

normal skin but shows increased melanin content in basal layer. It also shows occasional clear cells called Toker cells in the basal layer.

The luminal cells in the lobules are capable of producing milk**.**The contractile myoepithelial cells assist in milk ejection during lactation and also produces structural support to lobules.

Immunohistoochemically the luminal epithelial cells were positive for keratin, EMA, lactalbumin, GCDFP-15. Myoepithelial cells were positive for S-100, Smooth Musle Actin,calponin,caldesmon also shows nuclear reactivity for p63

PHYSIOLOGY OF BREAST:

Estrogen and progesterone plays a major role in the development of breast. During the first phase of menstrual cycle the lobules are relatively inactive. After ovulation, under the control of estrogen and increasing level of progesterone, proliferation of cells increases and the number of acini increases per lobule. The intralobular stroma will become edematous. During menstruation, as the estrogen and progesterone levels begins to fall there will be regression of the lobules with disappearance of the stromal edema.

During pregnancy the breast becomes completely mature and functional. There will be a progressive increase in number and size of the lobules which are separated by relatively scant stroma**.**After delivery, the luminal cells start producing colostrum**.** As the progesterone level drops in the next 10 days there will be milk secretion. On cessation of lactation, the

epithelial cells undergo apoptosis, the lobules regress and become atrophic. However, full regression will not occur. During premenopause, there will be involution of the lobules**.** In elderly females, the lobules may become completely atrophic.

BENIGN EPITHELIAL LESIONS:

A large number of benign lesions arising from both the ducts and lobules have been found in the breast. These include non-proliferative changes, proliferative breast lesions and proliferative breast diseases with atypia[16-17].

NON-PROLIFERATIVE CHANGES:

This group comprises lesions such as duct ectasia, simple cysts of breast, apocrine metaplasia, fibrosis, adenosis etc. of the breast. All these are termed as the fibrocystic changes of breast. These lesions contain small fragments of normal duct epithelial cells with a background of cyst fluid and cyst macrophages along with scattered bare bipolar nuclei[18].

CYSTS OF THE BREAST

Most of the palpable breast swellings are formed by single or multiple cysts in the breast. The fibrocystic diseases usually present as cystic swellings containing a single layer of cuboidal or flattened epithelial cells. At times, a papillary pattern may be found. The lumen of the cysts contain fluid composed of desquamated cells and cyst macrophages (large cells with vacuolated cytoplasm), also known as foam cells.

The cystic fluid also contains various numbers of benign duct epithelial cells which are most often poorly preserved. If papillary proliferation is seen in the cysts, the epithelial cells will be larger and in large numbers. Almost one-third of the cystic swellings are lined by large cells called apocrine cells, which are larger with abundant eosinophilic granular cytoplasm.

FIBROCYSTIC DISEASE:

Fibrocystic disease is one of the most common disorders of the female breast. Mature woman are most commonly affected especially during the period of pre-menopausal years. It involves all the three (ductal, lobular and stromal) elements of the breast. The disorder is also referred as fibroadenosis, cystic mammary dysplasia, benign mammary dysplasia, and benign cystic mastopathy. The involved ducts are dilated at various levels (duct ectasia), which show as cystic swellings containing fluid. The lobular ductules undergo hyperplastic proliferation (adenosis) and are surrounded by proliferating stroma (fibrosis).

In Sclerosing adenosis, a variant of fibroadenosis, the hyperplastic ductules are separated into tubules by dense collagenous bands of fibrous tissue. The lining epithelium may also be hypertrophied and multiplied (epitheliosis).They are usually diagnosed by the honey comb pattern of the sheets of benign duct epithelial cells and the presence of benign apocrine cells and foam cells in the background of scattered naked bipolar nuclei[16]. In

cases with marked duct ectasia, necrosis is seen and the foamy macrophages show inspissated secretions containing dark-staining nuclei and a “dirty” appearing cytoplasm[17].

The cellular composition of fibrocystic disease may vary**.**In cases of marked fibrosis, the dense collagenous tissue resists aspiration and hence the sample will be acellular. If adenosis is predominant, the smear reveals only epithelial cells. The cytological diagnosis thereby will be incomplete and inadequate.

Wellings and Alpers[18]observed that no apocrine metaplasia was seen in the patients between the ages of 13 and 19 while this change was found in almost half of the patients above 30 years of age.

PROLIFERATIVE BREAST LESIONS:

These are again subdivided into two groups proliferative breast diseases without / with atypia. Under the category of proliferative disease without atypia comes the moderate to florid epithelial hyperplasia (epitheliosis), complex sclerosing lesion (radial scar), papilloma, sclerosing adenosis. While under the category of proliferative disease with atypia comes the atypical ductal / lobular hyperplasia.

EPITHELIAL HYPERPLASIA:

The ducts and lobules of the breasts are normally lined by the two layers of epithelial and myoepithelial cells. When more than two layers of cells which may be of either luminal or myoepithelial cell type are seen, it is

termed as epithelial hyperplasia. These hyperplastic cells proliferate and distend the lobules and ducts leading to the formation of irregular lumens.

Cytologically, they are identified by the streaming pattern of arrangement of large slightly disorganised sheets of cohesive duct epithelial cells with focal crowding and overlapping of the nuclei, associated with mild nuclear atypia in a background of bare bipolar nuclei and foamy cells[19].

ATYPICAL DUCTAL HYPERPLASIA:

Atypical ductal hyperplasia is identical to that of low grade ductal carcinoma in situ in various entities like high cellular proliferation etc. Till now no single feature has been found with reliability to differentiate between them[20].

Cytologically, the smears are highly cellular and present typically in a cribriform pattern of large sheets of cohesive mildly atypical epithelial cells in a background showing naked bipolar and myoepithelial nuclei, necrotic debris and calcium granules.

DUCT PAPILLOMA:

The duct papilloma most commonly affects the main secretory ducts and present with a bloody discharge from the nipple. The epithelial lining of the affected ducts hypertrophies and projects into the cyst cavity as papillary projections which have a dense fibro vascular stromal core lined by epithelial cells showing mild nuclear atypia along with macrophages and

variable amount of cystic fluid in a background showing sparse naked bipolar nuclei[31]. Benign duct papillomas may be single or many in number[21].

INVASIVE DUCTAL CARCINOMA:

In 2nd century A.D Galen opined that "The breast carcinoma exactly resembles that of the animal crab". He also compared the veins that arose from the unnatural growth to that of the crab's legs[23] Rosen[24] (1979) suggested that invasive ductal carcinoma constituted nearly 75% of the deaths due to breast cancer. Currently, the Terminal Duct Lobular Unit (TDLU) is considered to be the site of origin for both invasive ductal as well as lobular carcinoma.

Azzopardi[25] et al also stated that most ductal carcinoma originate from the TDLU. Most of the breast malignancies that arise from ductal or lobular epithelium are adenocarcinomas. The predominant type among these are invasive ductal carcinomas (80%). Invasive ductal carcinoma of No Special Type (NST) constitutes the majority of the invasive ductal carcinomas (75%)[26,27].

Cytologically, they are highly cellular with neoplastic cells being arranged in irregular dyscohesive aggregates or sheets with large pleomorphic cells with malignant nuclear features in a background of nuclear debris and granular calcium[22].

The other types of primary breast carcinoma are as follows:

Lobular carcinoma, mucinous carcinoma, tubular carcinoma, medullary carcinoma, papillary carcinoma, clear cell carcinoma, secretory carcinoma, adenoid cystic carcinoma and metaplastic carcinoma.

INVASIVE DUCTAL CARCINOMA

Invasive ductal carcinoma are tumors in which stromal invasion is detectable**.**Regardless of the presence of in situ component and the relative proportion of in situ and invasive component they are included under invasive carcinoma.

Invasive carcinomas can be classified into two major categories-ductal and lobular type. Invasive ductal carcinoma comprises 75-85% of mammary carcinoma**.**Invasive ductal carcinoma,not otherwise specified comprises majority of duct carcinoma**.**Other relatively infrequent forms of infiltrating ductal carcinoma include tubular,medullary,metaplastic,colloid carcinoma etc.,[21]

CYTOARCHITECTURAL TYPES:

INVASIVE DUCTAL CARCINOMA, NOS TYPE:

IDC,NOS type comprises 75 % of all the cases of breast carcinoma. It represents the prototype of all breast carcinomas.[7]The tumor is usually a ill-circumscribed firm tumor.It shows a grayish yellow cut surface. The trabeculae radiates through the surrounding breast parenchyma in to the adjacent fat with a crab like or stellate configuration.

Figure 2 - Gross Appearance Of Invasive Ductal Carcinoma

In case of larger tumors areas of hemorrhage, necrosis and cystic degeneration may be present. In older days the term scirrhous carcinoma has been used for tumors with hard consistency. The hard consistency is due to presence of large amounts of stroma.

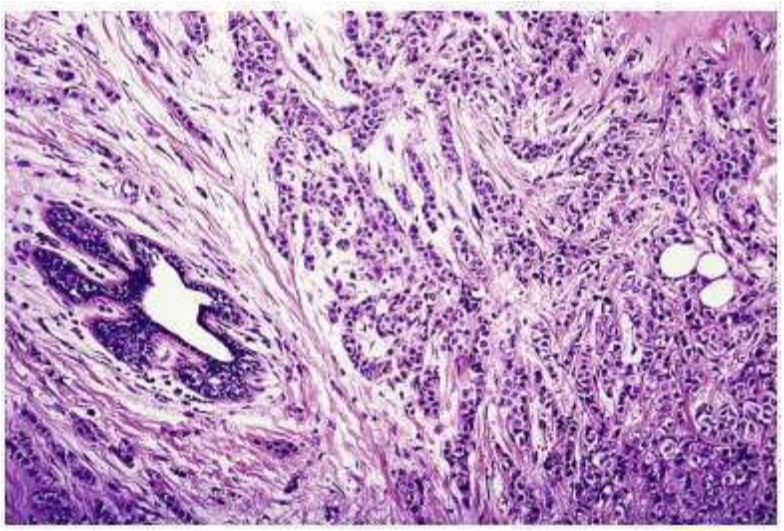

Fig-3 Microscopic Appearance Of Invasive Ductal Carcinoma

The tumor shows various growth pattern like diffuse sheets, nests,cords,trabeculae and also as individual cells.Glandular differentiation may be well developed to barely detectable. The individual tumor cells are usually large and pleomorphic compared to that of classical invasive lobular carcinoma.

The tumor shows prominent nuclei and nucleoli and increased mitotic figures. About 60% of the cases shows areas of necrosis.The amount of stroma varies from scant to abundant desmoplastic stroma. Elastic tissue are present in about 90% of cases. The presence of chalky streaks on gross examination is due to the presence of elastosis involving the vessel and duct walls**.**About 60% of the cases show calcification. The interphase between the tumor and stroma shows mononuclear cell inflammatory infiltrates.[8]

Studies done by Fisher et al. showed that lymphatic, blood vessel and perineural invasion was found in 33%, 5% and 28% of the cases[7a]. The tumor cells are positive for low molecular weight keratin (8,18 and 19) and EMA. Other sensitive breast related markers are mammoglobin and GCDFP 15.The basement membrane components collagen 4 and laminin shows a discontinuous linear pattern or it may be totally absent. [(8)]

INVASIVE CRIBRIFORM CARCINOMA:

Invasive cribriform carcinoma is a rare form of breast malignancy.

The tumor shows a cribriform appearance similar to that of its intraductal counterpart but in addition it shows stromal invasion. Cribriform

pattern is often seen in association with tubular formations. Page et al proposed that the relative proportion of the two elements determine the term to be used.

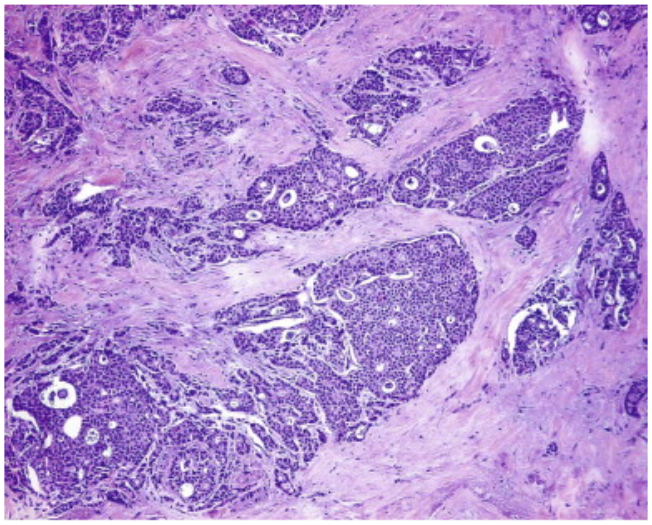

Fig 4 : **Microscopic Appearance Of Invasive Cribriform Carcinoma**

The tumor has an excellent prognosis.

TUBULAR CARCINOMA:

Pure tubular carcinoma comprises less than 2% of invasive breast cancer. But in mammographic screening 9-19 % of cases can be detected. It is easily detectable due to its speculate nature and cellular stroma.

The gross feature of tubular carcinoma is similar to that IDC, NOS type with poorly circumscribed margins and hard consistency. But the size of the tumor is usually small with a mean diameter of 1 cm.

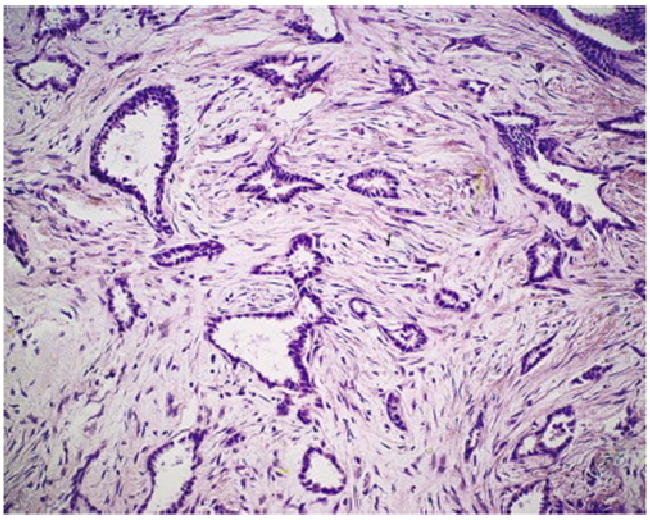

Fig 5 : Microscopic Appearance Of Tubular Carcinoma.

The tumor shows haphazard arrangement of glands without any organoid configuration. The characteristic feature of tubular carcinoma is irregular and angulated contour of the glands**.**The lining cells show apocrine type snouts in the apical cytoplasm.They lack myoepithelial cells and basement membrane. The lumina of the glands are open and filled with basophilic secretion. It shows a cellular desmoplastic stroma. The tumor is cellular with fat invasion in the periphery. Because of the well differentiated nature of the glands, scant pleomorphism and absence of necrosis it simulates benign conditions like microglandular adenosis,sclerosing adenosis and radial scar**.**[7-8] DCIS can be seen in majority of the cases. The in situ component is usually of low grade showing cribriform or papillary pattern.

Tubular pattern can be seen associated with invasive ductal carcinoma, NOS type or sometimes with invasive lobular carcinoma.In this instances,

the term tubular NOS and tubular mixed can be employed.When the tubular pattern is more than 75 %,the tumor shows better prognosis than ductal carcinoma NOS type. The term tubular carcinoma can be best employed for tumors in which tubular pattern is present for atleast 90 % of the tumor. These tumors are associated with favorable prognosis.

MUCINOUS CARCINOMA:

Mucinous carcinoma was classified under mucin producing carcinoma. Other mucin producing carcinomas are mucinous cystadenocarcinoma, columnar cell mucinous carcinoma and signet ring cell carcinoma.

The tumor usually occurs in postmenopausal women. It is also called as mucoid, gelatinous or colloid carcinoma.The tumor is well circumscribed**.**Cut surface of the tumour shows a characteristic glistening and gelatinous appearance.

Figure-6 Gross Picture Of Mucinous Carcinoma

The tumor cells are arranged usually in small clusters floating in a mucinous pool which are surrounded by bands of fibrous septa.The tumor cells show little pleomorphism.Mitotic rate is uaually low.The mucin is usually extracellular**.**The mucin may be acid or neutral type.The in situ component is usually difficult to recognize.

Histochemically, the mucins are o-acylated forms of sialomucin. Immuohistochemically there is strong MUC2 positivity in cytoplasm.Estrogen and progesterone receptors are always positive whereas Her 2 neu will be negative.

Few studies suggest that mucinous carcinoma can be classified as A and B based on the endocrine differentiation. Type A tumors shows trabeculae of malignant cells with minimal intracytoplasmic mucin. The cells do not show argyrophilia.Type B tumors showas sheets of tumor cells with abundant intracytoplasmic mucin. Argyrophilia can be demonstrated in the tumor cells.

Nodal Metastasis is very low which accounts for 2-4% of node metastasis.[8] They are positive for estrogen and progesterone receptors. They usually do not show HER2/neu overexpression or p53 accumulation.

MEDULLARY CARCINOMA:

The tumor is most common in patients under 50 years of age.The tumor is common among carriers of BRCA1 mutation. The tumor is well circumscribed, solid and homogenous.

The tumor grows in a diffuse pattern with minimal or absent glandular differentiation. The individual tumor cells are large, pleomorphic with large nuclei and prominent nucleoli.

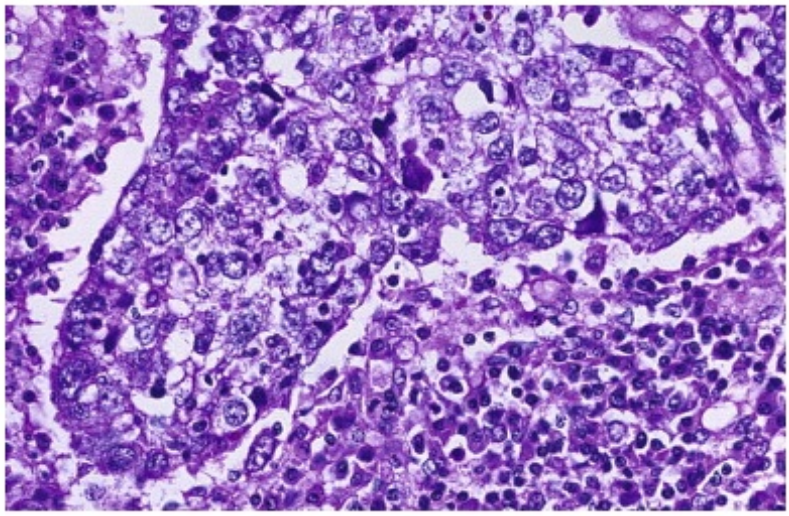

Figure 7: Microscopic Appearance Of Medullary Carcinoma.

The cell borders are indistinct which gives the tumor a syncytial arrangement.Spindle cell metaplasia, tumor giant cells and necrosis may occur.The tumor shows prominent lymphoplasmacytic infiltrate at the periphery of the tumor which is an characteristic feature of medullary carcinoma. The infiltrate was thought due to the reaction of host tissues to the neoplasm. They are usually peripheral T cell type.

They are positive for CK7, p53. They are negative for hormone receptors (ER,PR),Her2/ neu and comes under triple negative phenotype. The tumor expresses HLA-DR antigen which could the possible reason for the prominent lymphoplasmacytic infiltrate. Though axillary lymph node

involvement are common, only few and low axillary group of lymph nodes will be involved.The prognosis will be better than IDC, NOS type.[7-8].

ATYPICAL MEDULLARY CARCINOMA:

The tumor shows same growth pattern that of typical medullay carcinoma but lacks the classic microscopic features.

The tumor shows

Syncytial growth comprising > 75% of the tumor

Atypical features

Focal tumor infiltration at the margins

Uniform nuclei and rare mitosis

Mild to absent lymphoplasmocytic infiltration at the margins.

Focal tubule formation. [7-8]

INVASIVE PAPILLARY CARCINOMA:

The tumor is rare and occurs more frequently in the postmenopausal women. Most commonly papillary carcinomas present as in situ lesions. The invasive component can be papillary or it may show features of IDC, NOS type. As the presence of invasion in these tumors are not clearcut, it should be applied for cases only with well differentiated true papillary structures. When a tumor with papillary pattern is seen, metastatic papillary carcinoma from other sites should also be excluded.

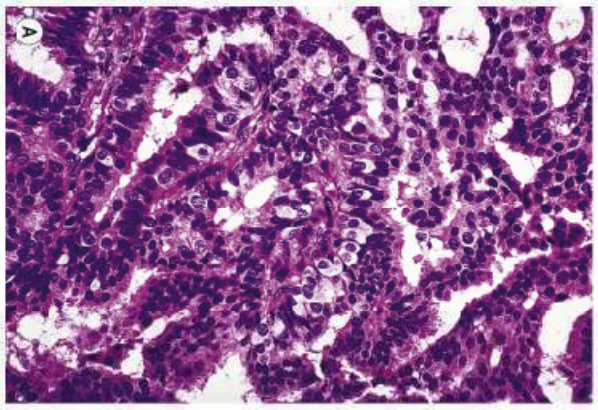

Fig -8 Invasive Papillary Carcinoma

The tumor may have axillary lymph node metastasis particularly in solid variant of papillary carcinoma. Prognosis of the tumor is better compared to that of invasive ductal carcinoma, NOS type.[7-8]

INVASIVE MICROPAPILLARY CARCINOMA:

Invasive miropapillary carcinoma is a distinct rare variant of invasive ductal carcinoma. When the micropapillary pattern is found throughout the tumor it is referred as pure invasive micropapillary carcinoma. When it is present as a part of conventional IDC it is called as mixed invasive micropapillary carcinoma**.**But the criteria to distinguish this two is not clear cut. Some authors suggest atleast 50 % of the tumor should be micropapillary to call it as pure invasive micropapillary carcinoma.

The tumor is composed of clusters of cells arranged in micropapillary or tubular pattern.The tumor cells are found free floating in clear spaces. Fibrovascular core will be absent in the micropapillary clusters. The clusters

exhibit a “inside out” arrangement. The apical cells are polarized outside and this can be evidenced by MUC 1 staining.

The nuclear grade of this tumor cells will be high. About half of the cases may show psammoma bodies. In situ component seen in these cases is usually micropapillary and sometimes cribriform pattern.

Lymphatic invasion was reported in more than 50 % of the cases.Lymph node metastasis usually occur.The tumor have a bad prognosis.[7-8]

Estrogen receptor were positive in 72-75 % of cases and 45 % cases were positive for progesterone receptor.36 % of the cases show Her 2-neu overexpression.

APOCRINE CARCINOMA:

Apocrine carcinoma is very rare comprising 0.5 % of all breast carcinoma. The tumor is composed entirely or predominantly of apocrine type cells.The tumor cells are large with abundant eosinophilic cytoplasm with vesicular nucleus and prominent nucleoli. Glandular differentiation can be seen with apocrine snouts. Diagnosis of apocrine carcinoma should only be made when the architectural features are those of a malignant tumor. Immunohistochemically they are positive for GCDFP-15.Estrogen and progesterone receptors will be negative. [7-8]

SECRETORY CARCINOMA:

Secretory carcinoma are rare tumors and seen in children. It can also occurs in adults. It has a excellent prognosis. The tumors are usually small and well circumscribed.

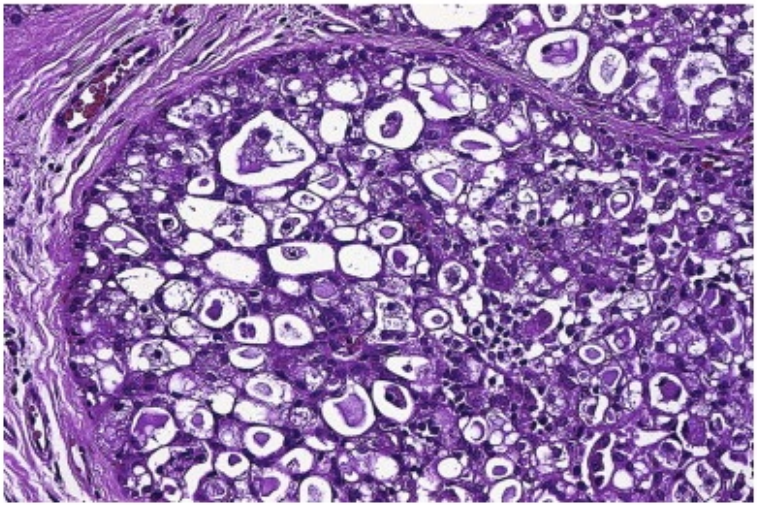

Figure 9:Microscopic Appearance Of Secretory Carcinoma

The tumor is composed of tubuloalveolar and papillary structures. The lumina contain eosinophilic PAS positive, diastase resistant material. The malignant cells have a pale staining vacuolated cytoplasm**.**Nucleoli may be prominent. Mitosis is scanty.

There is a strong immunoreactivity for S-100 and a-lactalbumin.

NEUROENDOCRINE TUMORS:

Primary neuroendocrine tumors of breast represent tumors which shows morphological features similar to neuroendocrine tumors of other sites. In these tumors more than 50 % of the cell population express

neuroendocrine markers**.**This group doesnot include invasive carcinoma, NOS type which shows focal neuroendocrine differentiation.

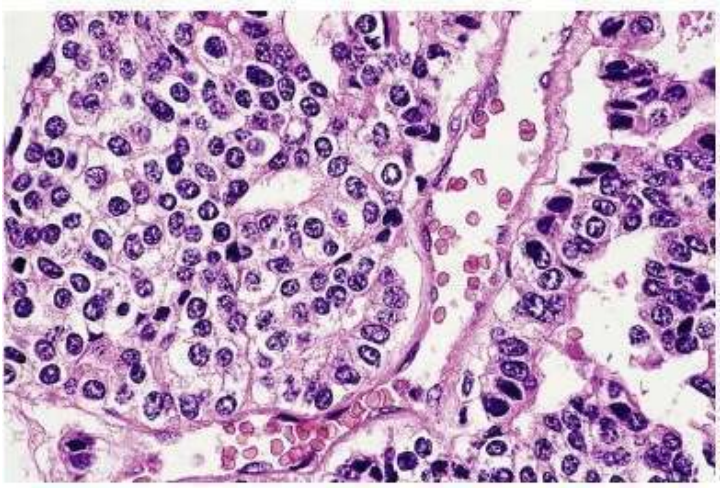

Figure 10- Breast Carcinoma With Neuroendocrine Differentiation

Neuroendocrine tumors of breast include

- solid neuroendocrine carcinoma
- atypical carcinoid tumor
- small cell carcinoma
- large cell carcinoma

The clinical presentation is similar to that of invasive ductal carcinoma. Bilaterality and multicentricity can occur.

Grossly the tumors has no distinctive features.

In most of the neuroendocrine tumors, the cells are arranged as solid nests or alveolar structures separated by fibrous tissue. The tumor cells are usually small in size. In large cell carcinoma, the tumor is composed of large clusters of cells with moderate to abundant cytoplasm, vesicular nucleus and

high mitotic rate. Minority of the cases show intraductal component and mucin secretion.

The tumor cells are argyrophilic**.** Ultrastructurally, they contain dense core secretory granules. Immnohistochemically, they are positive for chromogranin, synaptophysin and neuron specific enolase. Apart from carcinoid tumors many mucinous carcinomas[48], few in situ and invasive lobular carcinoma are also argyrophilic.. Studies done by Upalakalin et al. found that among carcinoid tumors 21 % were metastasis from intestine, lung[8a].

Estrogen and progesterone are found positive.[(7-8)]

METAPLASTIC CARCINOMA:

Metaplastic carcinoma represents tumor predominantly with cell type other than epithelial and glandular component. It includes many categories but which overlap with each other. Metaplastic carcinoma is more aggressive than invasive ductal carcinoma.Metastasis is usually hematogenous rather than lymph node metastasis.[(7-8)]

The tumors are circumscribed, firm to hard in consistency. Degenerated cystic areas can be seen in cases with squamous metaplasia. Some of the tumors may have infiltrative borders.

A.**Squamous cell carcinoma:**

The tumors are large with cystic spaces filled with keratin.

In pure squamous cell carcinoma the central cystic cavity is lined by malignant squamous cells. Most cases represent squamous metaplasia**.**

Other two variants which can be seen will be acantholytic squamous cell carcinoma and adenosquamous carcinoma. Low grade adenosquamous carcinoma is said to have a favourable prognosis whereas acantholytic squamous cell carcinoma have a aggressive behavior.

B.**Carcinosarcoma:**

Microscopically the sarcoma like component can be malignant fibrous histiocytoma, osteosarcoma, chondrosarcoma, angiosarcoma or a combination of various components.When the transition between sarcomatous and carcinomatous component is gradual and sharp, it is termed carcinosarcoma.

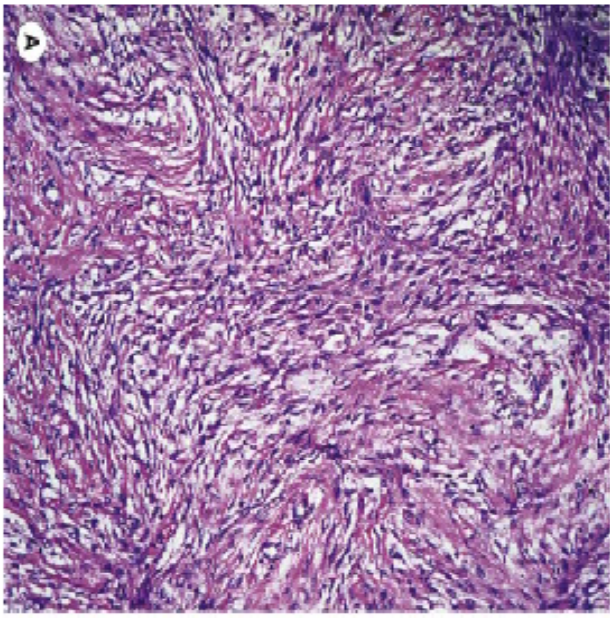

Figure 11- Microscopic Appearance Of Metaplastic Carcinoma

When the transition to osseous or cartilaginous elements is direct without any intervening spindle cell component or osteoclastic giant cells,it is called matrix producing carcinoma.

Molecular studies suggest that the epithelial and sarcoma like components originate from same stem cell.

The sarcoma like elements acquire vimentin and other mesenchymal features**.**It is referred to as the phenotypic switch.The cells are occasionally positive for epithelial markers.

PAGETS DISEASE:

Pagets disease was originally described by Sir James Paget in 1874. It is a crusted lesion of nipple caused by underlying breast carcinoma. About 1-2% of patients with mammary carcinoma show pagets disease. The accompanying breast carcinoma is usually a intraductal carcinoma**.**It may be associated with or without stromal invasion. The epidermis of the nipple shows characteristic of Paget's cells in the keratinizing epithelium. The cells may be singly scattered in the superficial epidermis. They may also form clusters in the basal portions of the epidermis. Individual cells have a pale or clear cytoplasm and their nuclei have a prominent nucleoli.

Intraductal carcinoma is usually of comedo or solid growth pattern.About 10 % of the cases show cribriform or papillary carcinoma and 40 % the cases show mixed type of in situ carcinoma.

79-100 % the cases are strongly positive for Her 2/neu.The underlying insitu lesions are frequently Her2 /neu.[7-8]

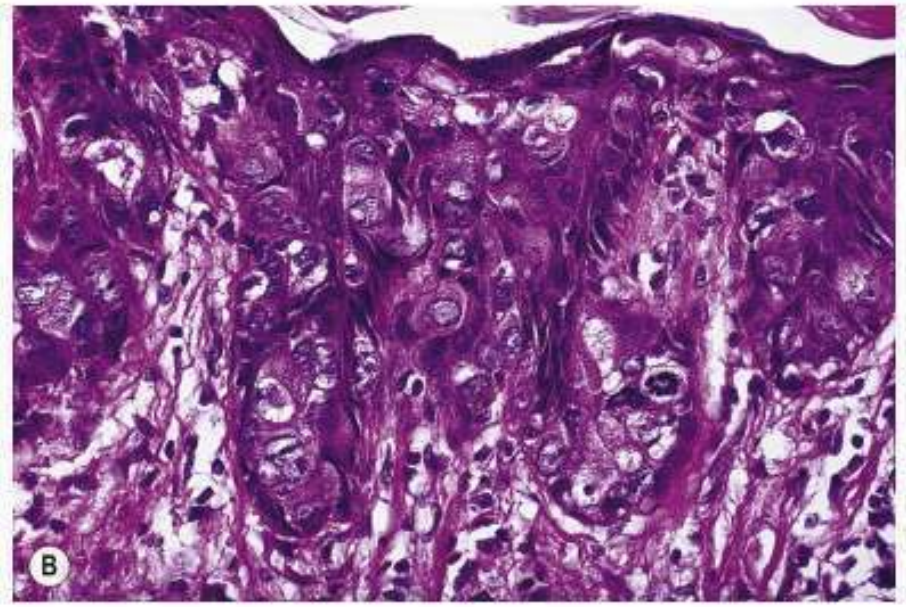

Figure 12- Pagets Disease Of Nipple

INFLAMMATORY CARCINOMA :

The diagnosis of inflammatory carcinoma is essentially based on clinical criteria. Clinically, the entire breast is red, warm. The skin shows widespread edema which resembles that of mastitis. It has been believed that clinical appearance is due to widespread carcinomatosis of dermal lymphatic vessels. Skin biopsy is usually performed to reveal dermal lymphatic permeation. Histopathological examination of some of the cases show a undifferentiated carcinoma.

The prognosis is usually bad. Studies done by Charafe-Jauffret et al. found most inflammatory carcinoma are negative for estrogen and positive for MIB1,E-Cadherin and Her2 neu.[7-8].

RISK FACTORS

Several risk factors have been established for the development of breast carcinoma**.** The common denominator for most of the factors will be strong and prolonged estrogen stimulation in a genetically susceptible background.

AGE:

Majority of the breast cancers are detected during the reproductive age group.[28]

RACE AND ETHNICITY:

The incidence is high in northern Europe and North America (91.4 new cases per 100 000 women/year), intermediate in southern European and low in African and asian countries. In the United States due to increased mammographic screening there has been a increase in the detection of breast carcinoma.[29] Due to earlier diagnosis and improved therapy the mortality has began to fall in some regions like North America, western Europe, and Australia.[29]

FAMILY HISTORY:

The risk is 2-3 times higher than general population if the first degree relative of the women had breast cancer**.**[30]

MENSTRUAL HISTORY:

Early menarche and late menopause is associated with increased risk of carcinoma.[30]

PARITY:

There will be a increased risk in case of Nulliparity and late age at first child birth. It has been documented that there is a reduced risk of breast carcinoma in premenopausal women who have lactated.[29]

HORMONE REPLACEMENT THERAPY:

Many studies showed that there is an increased risk of breast carcinoma in women under hormone replacement therapy than women using estrogen alone. [(32)]

RADIATION:

An increased risk of breast cancer has been documented on exposure to ionizing radiation particularly when the exposure is at the time of breast development.[(36)]

PRECANCEROUS LESION:

Complex fibroadenoma,florid hyperplasia without atypia,solitary papilloma without atypical hyperplasia, sclerosing adenosis are associated with a risk of 1.5-2 times than that of general population**.**Atypical ductal hyperplasia and atypical lobular hyperplasia are associated with a risk of 4.0-5.0 than that of general population.[33]

BREAST DENSITY :

Breast density solely and strongly contributes to the risk of breast cancer[32].

GENETIC PREDISPOSITION:

About 5-10 % of breast cancers are familial.[33] In various studies it has been reported that the risk of developing breast carcinoma due to BRCA1 mutation will be 56% to 90%**.**Women carrying BRCA2 mutation have a risk of 37% to 84%**.**BRCA1 mutation may account for about 45% of cases of hereditary breast carcinoma and they are usually of poorly differentiated type with high proliferative rate.[33]

MODIFIABLE RISK FACTORS

BODY MASS INDEX(diet)

Western diet (High caloric diet rich in proteins and animal fat),obesity[30],,sedentary lifestyle are associated with increased risk of breast carcinoma[34].

ALCOHOL CONSUMPTION:

Increased alcohol consumption associated with increased risk of breast carcinomas[30,35].

PROGNOSTIC AND PREDICTIVE FACTORS:

A predictive factor is the one that gives information about the response of a tumour to a specific treatment.

A prognostic factor gives information about the recurrence risk of the tumour in the absence of the adjuvant therapy, that is a prognostic factor is a factor that helps to predict the natural history of a tumour.

Many prognostic and predictive factors help in the process of estimation of risk for recurrence and in selection of additional therapies.

AGE :

Women less than 50 years have the best prognosis. Older patients have a higher rate of recurrence and distant metastasis[7].

PREGNANCY:

Carcinoma breast manifesting during pregnancy may enhance tumour angiogenesis, over expression of Her 2 neu, and low expression of hormone receptors. The tumors are generally aggressive in these cases.[7]

GENE:

Studies showed that BRCA1 mutation carriers have a low survival rate.

SKIN AND NIPPLE INVASION:

Invasion of overlying skin is associated with decreased survival rate. Nipple involvement is associated with high incidence of axillary node metastasis

PRESENCE OR ABSENCE OF INVASION:

The single most important prognostic determinator is the presence of invasive component. In case of a tumor with both an invasive and in situ component,the invasive component is proportional to the nodal

metastasis.The in situ component is directly related to the incidence of multicentricity and indirectly with occult metastasis.

HISTOLOGICAL TYPE OF TUMOUR:

Variants of invasive ductal carcinoma like tubular carcinoma, cribriform carcinoma, medullary carcinoma, papillary carcinoma, pure mucinous carcinoma and secretoty carcinoma have a more favorable prognosis than Invasive ductal carcinoma,NOS type. Signet ring cell carcinoma have a bad prognosis.

NECROSIS:

Spontaneous tumor necrosis is associated with tumors showing high histological grade and increased incidence of lymph node metastases.[7]

LYMPHOVASCULAR INVASION:

Lymphatic emboli within the breast is associated with higher tumor recurrence. [58]Vascular emboli is highly correlated with tumor size, tumor type, histological grade, lymph status and have a poor prognosis.

LYMPH NODE STATUS AND TUMOUR SIZE:

For a woman with operable breast cancer the presence of lymph node metastases is the strongest prognostic factor [37]. The pN staging is a qualitative parameter.

The prognostic value of lymphnode status can be increased by counting the number of lymph nodes. This has been shown in an study done on more than 24,000 women in American population [38]. A minium of 10 lymphnodes should be examined to correctly establish pN status [39]. The

quality of the procedure by counting the number of examined lymph nodes depends on both the surgeon and the pathologist [40].

The tumour size is also another useful prognostic factor and can be obtained in almost all cases. Thus, recurrence risk has been correlated with tumour size [41, 42,].

TNM N0	Stage 1	No node involvement
TNM N1	Stage 2	Upto 3 axillary/single internal mammary node involved
TNM N2	Stage 3	4 or more axillary node involved
TNM N3	Stage 3	>10 axillary node involved.

TUMOUR GRADE:

The current grading system in use was first proposed by Bloom and Richardson[43] and thereafter it was reclassified by Elston and Ellis [44].

HISTOLOGICAL GRADING SYSTEM:

Tumor grade is describing the tumor on the basis of how much abnormal the tumor cells and the tumor tissue look under a microscope. Bloom and Richardson histological(43) grading method is most commonly used for grading the breast cancer. This method is very simple and gives very good prognostic implications. This system grades breast cancer into three types on the basis of the following three criteria:

i. measurement of tubular differentiation
ii. nuclear pleomorphism, and

iii. proliferative index.

TABLE -1: MODIFIED BLOOM AND RICHARDSON GRADING SYSTEM

PARAMETER	CRITERIA	SCORE
Tubule formation	>75%	1
	10-75%	2
	<10%	3
Nuclear pleomorphism	Small and uniform	1
	Moderate variation	2
	Marked variation	3
Mitotic count/hpf	0-5	1
	6-10	2
	10-15	3

TABLE-2: BLOOM RICHARDSON GRADING SYSTEM

GRADES	SCORE
Grade -1(well differentiated)	3-5
Grade-2(moderately differentiated)	6-7
Grade-3(poorly differentiated)	8-9

Added score Tumour grade Degree of differentiation

- 3-5 Grade I Well differentiated
- 6-7 Grade II Moderately differentiated
- 8-9 Grade III Poorly differentiated

According to Elston and Ellis tumour grade is a well developed prognostic indicator for overall survival of breast cancer patients [44, 45]. The estimation of histologic grade is simple and cheap, so that it can be done in almost all woman with breast carcinoma. One of the disadvantages is that the estimation of histologic grade usually varies among the observers.

Boiesen and co-writers[46] were performed histologic grading on ninty three breast carcinomas patients in seven pathologic departments, results showed an mean kappa value of 0 and 54, which indicates a moderate level of reproducibility.

ESTROGEN- AND PROGESTERONE RECEPTOR STATUS :

Studies found that a vast majority of breast cancer tumours expressed oestrogen receptors and progesterone receptors**.** The first study on the prognostic importance of estrogen receptor was done almost 20 years ago [49]. However, estrogen receptor and progesterone receptor are not strong prognostic indicators with longer follow-up time, though women with positive receptor breast carcinomas have a good prognosis compared to the receptor negative breast cancer[50].

Only in the late 1970ies, the value of estrogen receptor and progesterone receptor as predictive factors was demonstrated [51].

Tumours with both estrogen and progesterone receptor positive are benefit more from hormonal therapy than those who have either estrogen or progesterone receptors [52].

Approximately 50-60% of women with positive receptor late stage breast cancer will have better response from hormonal therapy than receptor negative women [53].

Currently, the biochemical analyses of estrogen receptor and progesterone receptor are largely replaced by immunohistochemistry**.** Immunohistochemistry gives superior prognostic information and it is more valuable for small tumours [53-54].

DNA CONTENT AND CELL PROLIFERATION MEASUREMENT:

DNA-PLOIDY:

DNA-ploidy is a measure of the deoxy ribonucleic acid content within the nucleus of somatic and germ cells. The normal cells are either diploid or euploid.

Measurement of DNA content within tumour cells has been applied to evaluation of number of tumour types. Bagwell et al showed that ploidy analysis may be useful prognostic test for node negative breast carcinoma[47].

Flow cytometry is the most common method used to measure the DNA content in cell nuclei.

Due to genetic instability most of the cancerous cells have a abnormal amount of DNA which may greater or lesser than that of actual amount of DNA.It is usually associated with a worse prognosis and this is called aneuploidy [47].

S-PHASE FRACTION (SPF):

The S-phase of DNA-synthesis takes place before cell division and hence it measures the proliferative activity of the cell. The number of cells in S-phase can be estimated by means of flow cytometry [48]. Most of the studies revealed that high SPF associates with greater recurrence risk of breast cancer, and is considered as a prognostic factor.

NEW PROGNOSTIC AND PREDICTIVE FACTORS:

The recently recognised prognostic and predictive markers provides more reliable information on prognosis and treatment selection**.**

A prognostic factor provides information on the outcome, irrespective of the different therapies employed. Whereas, a predictive factor gives valuable information on the likely benefit from a particular treatment.

So far, the sex-hormone receptor status is the only well established predictive factor useful in the management of patients with breast carcinoma.

Therefore,an increase in need for other markers, especially prognostic- and predictive factors employing at the individual level.

Indeed, many studies were conducted to identify different risk groups based on certain prognostic factors [55].

In recent years, there are three emerging predictive and prognostic factors of special interest as follows,

- c-erbB-2 gene,
- p53 gene and
- angiogenesis.

One of the markers, c-erbB-2 oncogene is a routinely used predictive factor, while p53 tumour suppressor gene and angiogenesis in the future may be of value in management of breast cancer.

c-erbB-2:

c-erbB-2 is an oncogene situated on the q arm of 17^{th} chromosome [55]., which encodes a tyrosine growth factor receptor belongs to EGFR(epidermal growth factor receptor). [56].

In a Swedish population based study showed that the prevalence of overexpression of c-erbB-2 was 19% among the breast cancer patients.[57]. Most of the studies revealed that overexpression of c- erB-2 gene has been associated with bad prognosis(57,58), though some studies have found that there is a strong association between over expression of c-erB-2 and reduced sensitivity to chemotherapy and tamoxifen.(60-61)

As a result, evaluation of c-erbB-2 status with immunohistochemistry on tumour sections has become a routine analysis for women with breast cancer.

p53:

p53 is a tumour 48uppressor gene situated on the p arm of 17th chromosome [59,62]. Because of its crucial role in control of cell cycle, apoptosis and DNA repair mechanism, p53 is rightly known as "the policeman of the genome" [59].

P53 Mutation is identified nearly 20-25% of breast carcinomas [63].

The commonest method of determining p53 protein is immunohistochemistry on tumour sections. This method detects accumulation of abnormal p53 protein with in the cell and also estimates the levels of normal p53 protein.

The potential use of p53 in the future seems to be as a predictive factor. Patients with p53 mutations found to reduced response to tamoxifen [64], whereas taxane based chemotherapy have been associated with greater efficacy in people with p53 mutated breast carcinomas. [65].

Hence, in future, evaluation of p53 status has one of the powerful markers to ameliorate the management of breast carcinoma**.**

ANGIOGENESIS:

The ability of a tumour to induce formation of new microvessels called angiogenesis, more than 30 years ago its importance for tumour growth was reported [69].

Angiogenesis induces the growth of both primary and metastatic tumours by different types of mechanisms.

- Tumour growth more than 1-2 mm^2 size is depends on tumour angiogenesis [64].In the absence of tumour microvessels, tumour growth is restricted by reduction of nutrients and accumulation of waste products.
- The tumour microvessels permits tumour cells to invade in to blood stream[69, 70], paved an initial pathway for distant metastases.
- Endothelial cells in the tumour microvessels releases growth factors which induce further growth of the tumour [68].Tumour neovascularisation also involved in the synthesis of various proteolytic enzymes [74]

Tumour angiogenesis is regulated by large number of pro angiogenic and antiangiogenic factors, vascular endothelial growth factor(VEGF) is one of the most important regulating factor [75].

VEGF act as a mutagen for tumour endothelial cells.

High levels of vascular endothelial growth factors are associated with bad prognosis in breast carcinoma. [76-81].

The factors that regulating the angiogenesis are tabulated as follows:

TABLE-3 FACTORS REGULATING ANGIOGENISIS :

PROANGIOGENIC FACTORS	ANTI ANGIOGENIC FACTORS
Vascular endothelial growth factor	angiopoeitin
Fibroblast growth factor	angiostatin
platelet derived growth factor	endostatin
Plasminogen activator inhibitor	thrombospondin
Angiogenin	Platelet factor 4
Plasminogen activators	Prolactin
Transforming growth factor	osteopontin

Interestingly, Linderholm and co-workers, revealed that p53 mutation associated with high levels of vascular endothelial growth factors. so, the p53 gene exerts a inhibitory effect on tumour angiogenesis, wild type p53 protein found to be inhibit the formation of VEGF(vascular endothelial growth factor)[82,83].

It has been showed that wild-type p53 protein upregulates the thrombospondin1, a strong antiangiogenic factor of angiogenesis [83].

So, when mutant p53 protein is present, causes inhibitory effects on thrombospondin 1 and subsequently increased tumour angiogenesis [83, 84].

Evaluation of tumour angiogenesis by a histological grading system and microvessel density grade has been correlates with certain clinico pathological entities like tumour size, tumour grade was described 30 years ago [85].

Weidner and co-workers were the first proposed the method for estimation of tumour angiogenesis [17]. They revealed that tumour microvessels can be highlighted with help of immunohistochemistry, by staining the tumour sections with an antibody against antigen expressed by tumour endothelial cells. The microvessel density is then evaluated by counting these identified microvessels within the region with the most intense new blood vessels, also called hotspots.

Most of the studies proved that microvessels density can predict bad prognosis in breast carcinoma. [86-90] including lymph-node negative patients [90-96].Whereas, some researchers could not find any prognostic significance of tumour microvessels density [97-101]. The reason for this results variation may be due to diferrent methods were used to estimate microvessels density.

Indeed, some studies reported contradictory results in the association between microvessels density and prognosis. However, most of the studies showed that there is a strong association between high microvessels density and metastases.

Although some studies reported poor correlation between microvessel density and lymph node metastases [102-103],, this relationship is of interest as the factors that associated with lymph-node metastases could be used for prediction of lymph-node metastases.

Thus a predictive factor is useful in identifying woman with low risk of metastases to avoid unnecessary axillary surgery and also defining the woman with high risk of lymph node metastases.

Moreover, the interest in the therapeutical aspect of angiogenesis has been focused on the development of specific anti-angiogenic drugs [104, 105]although there have been some reports on the predictive value of angiogenesis [106-110] but in search for a new class of antiangiogenic drugs is still to be awaited.

TYPES OF ANGIOGENESIS:

There are 2 types of angiogenesis, they are

1. Intussusceptive angiogenesis
2. Sprouting angiogenesis

INTUSSUSCEPTIVE ANGIOGENESIS:

Intussusceptive angiogenesis is otherwise known as splitting type of angiogenesis because it splits the vessels in two by direct extension of the vessel wall in to the lumen of the already preexisting vessels.

It is very fast and efficient compared with sprouting angiogenesis because it requires only rearragement of already pre existing endothelial cells and does not depends on endothelial cell migration and proliferation.

Intussusceptive angiogenesis plays an essential role in formation of new vessels in embryos where resources are limited and growth is fast [111-112]. Its occurrence is based upon the formation of tissue pillars**.**

Scanning electron microscopes and three-dimensional reconstruction of serial microscopes are useful in identification of transcapillary tissue pillars.

This type of angiogenesis was discovered in postnatal lungs of rats and humans [113,114), but it also occurs in other tissues and organs like choroid of the eye, intestinal mucosa, kidney, ovary, and uterus [115,116]. It also occurs in skeletal muscle, heart, and brain.

It plays an important role in the formation of vein and artery bifurcations and also pruning of larger microvessels. Intussusceptive angiogenesis can be identified by determining the frequency of tissue pillars from scanning electron micrographs of vascular casts[116]**.**

SPROUTING ANGIOGENESIS:

The first discovery of sprouting angiogenesis in growth of the tumour was reported by Ausprunk and Folkman, 1977, which consists of following steps:

i. The basement membrane is locally degraded on the side of the peritumoral postcapillary venule situated nearer to the angiogenic stimuli;

ii. Cell to cell contacts between endothelial cells are loosened and endothelial cells transmigrate into the surrounding extracellular connective tissue matrix;

iii. Endothelial cells in the extracellular connective tissue matrix form solid cord;

iv. Finally, The formation of lumen takes place infront of the migrating portion, newly tubular spuds contact each other and joins to form functional capillaries, along basement membrane synthesis and rimming of pericytes around basement membrane.

Ausprunk and Folkman were first to proposed that tumour vessel spuding can advanced without cell division in the early stage of angiogeneis, while further outgrowth and prolonged sprouting requires proliferation of endothelial cells.

A large number of in vivo and in vitro models of angiogenesis have been introduced with the advancement in this field. In in vitro assays, the most common methods in use are,

i. rabbit cornea by Muthukkaruppan and Auerbach, 1979[117],

ii. developing mouse retina by Fruttiger, 2007[118], and

iii. intersegmental vessel growth in zebrafish by Lawson and Weinstein, 2002[119,120].

Cimpean et al in 2010[121] has studied selected aspects of the angiogenesis with an isolated cell lines which includes endothelial cell migration,endothelial cell proliferation, degradation of the connective tissue matrix and formation of capillaries.

Grant et al[122], first demonstrated the 3 dimensional structure of capillary networks and highlighted the membranes of endothelial cells with help of light and electron microscopes.

In1991, Paku and Paweletz [123], they proposed steps in sprouting angiogenesis by integrated with the studies of Ausprunk and Folkman, as follows:

1. A structural change in the basement membrane takes place, there is a loss of polarity along the whole surface of the dilated blood vessel, followed by a loss of the basement membrane at site where endothelial cell processes are protruding into the extracellular connective tissue;
2. Endothelial cells are lined up in parallel manner, forming a small lumen and maintaining their basal-luminal polarity by intact endothelial junctions;
3. The polarized endothelial cells are continuously synthesis basement membrane all over the sprouting blood vessels, except at the tip of developing capillary bud,

4. Thereafter, pericytes are recruited over the basement membrane of the developing capillaries,
5. Finally, the growing capillary bud transforms in to a new vessel.

Accordingly to this model, 2 types of cells are involved in sprouting angiogenesis,

1. “tip cell”
2. “stalk cell”.

Tip cells are polarized and migratory in nature. Stalk cell undergoes proliferation during capillary sprouting and forms the new lumen of the blood vessels.

The phenotypic differentiation between tip cells and stalk cells is not permanent and reversible, it depends on the balance between pro-angiogenic factors like vascular endothelial growth factor and Jagged-1, and anti angiogenic factors like delta-like ligand 4-Notch activity,as proposed by Eilken and Adams in 2010, Geudens and Gerhardt in 2011, Wacker and Gerhardt et al 2011 [124,125,126,127].

Tip cells usually express high levels of delta-like ligand 4, platelet derived growth factor-b (PDGF-b), unc-5 homolog b (UNC5b), VEGF receptor-2 (VEGFR-2), and VEGFR-3/Flt-4, and have low levels of Notch signaling activity according to Gerhardt et al., 2003[128].

The tip cell extend enormous filopodia, these structures guide the newly formed blood vessel in a particular pathway toward an stimulus,

further it divides and multiplies and while moving it adopts a complex branched structure.

Stalk cells have lesser filopodia than tip cells. They are highly proliferative than tip cells, divides in to vascular lumen and branches. They also form junctions with adjacent endothelial cells and produce substances of basement membrane materials. (Phng and Gerhardt, 2009)[128].

During the transformation from active to inactive endothelial cells tip cell transformed in to a "phalanx" phenotype, which resembles like a phalanx formation of Greek soldiers, that is static cells, which stabilizes the blood vessels through tight cell to cell attachment and promotes the integrity of blood vessels and also resists the action of VEGF(vascular endothelial growth factor). Bautch, 2009 and Mazzone et al., 2009[129].

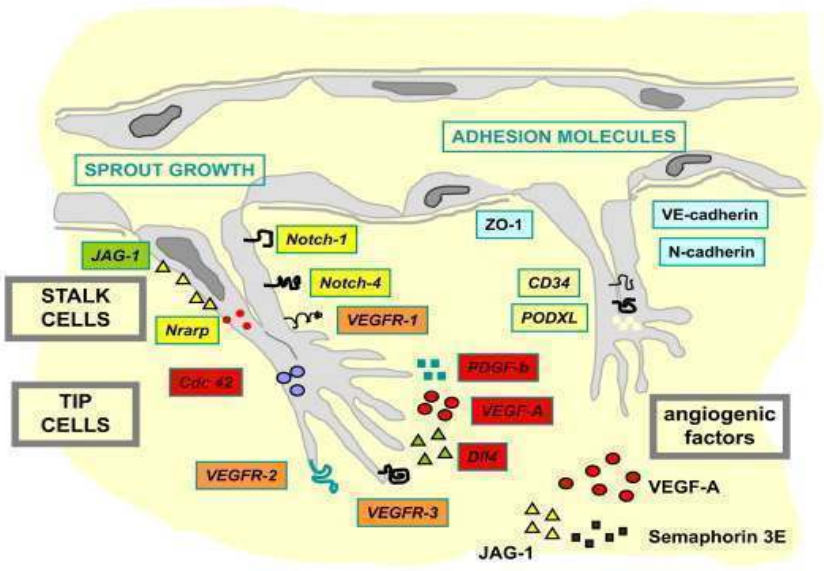

Fig 13 : Molecular Mechanism Of Sprouting Angiogenesis

TUMOUR ANGIOGENESIS:

Angiogenesis is a process involving interaction between endothelial cells, angiogenic factors, and extracellular matrix.

For the formation of new capillaries, endothelial cells of existing blood vessels should degrade the basement membrane and enter into the stroma of the surrounding tissue**.** These processes of invasion of endothelial cells and migration needed a integrative activity between proangiogenic system and the matrix metallo protease system.

1. The first group of proangiogenic factors are vascular endothelial growth factor family and the angiopoietins, which directly act on endothelial cells.
2. The second group of factors consists of directly acting substances, which includes many chemokines, cytokines, and angiogenic enzymes that activate a large number of target cells**.**
3. The third class of angiogenic factors are indirectly acting molecules, their effects are results from the release of directly acting substances from endothelial cells, macrophages, and tumor cells.

Thus, Tumor angiogenesis is a network of proliferation of blood vessels that invades into tumour, providing nutrients and oxygen and draining waste substances, has been recognised as one of the hallmark of carcinoma -Hanahan and Weinberg, 2011[(105)].

Tumor growth and metastasis depend on angiogenesis and lymphangiogenesis stimulated by chemical signals from tumor cells in the rapid growth phase Folkman 1971et al[130].

In a study, muthukaruppan and colleagues[117] were analysed the behavior of tumour cells injected into different foci of the same organ. The tumour cells devoid of blood circulation grew only up to a diameter of 1–2 mm^3 and but when placed in an area where angiogenesis taken place, it grew more than 2 mm^3 of diameter.

Holmgren et al 1995[131] and Parangi et al 1996[132] showed that in absence of angiogenesis, tumors may become necrotic**.** Therefore, angiogenesis is an important factor in the tumour growth.

There are four steps in development of tumour angiogenesis, they are as follows,

1. The basement membrane of endothelial cells is locally injured.
2. Endothelial cells activated by pro angiogenic factors and migrate in to the area of neovascularisation**.**
3. Endothelial cells proliferate and stabilize.
4. Proangiogenic and anti angiogenic factors continue to regulate the whole angiogenic process.

According to denekemp et al, endothelial cells in the mature blood vessels proliferates at a rate of every 1000 days on average[133]**.**

Thus, the angiogenesis is stimulated when tumor tissues require nutrients and oxygen. Angiogenesis is controlled by both activator and inhibitor molecules.

IMMUNOHISTO CHEMISTRY IN DETECTION OF NEOVASCULARISATION:

IMMUNOHISTOCHEMISTRY :

The principle of IHC has been known since the 1930s, but it was only in 1942 that the first Immunohistochemistry study was reported. Coons et al in 1942, first used FITC-labeled antibodies to detect Pneumococcal antigens in infected tissue.

IHC is used for diagnosis of disease, development of drugs and biological research. Using specific tumor markers, clinicians use Immunohistochemistry to diagnose a cancer as benign or malignant, determine the stage and grade of a tumor, and identify the cell type and origin of a metastasis to find the site of the primary tumor.

Immunohistochemistry (IHC) or immunocytochemistry is the application of immunologic principles and techniques to demonstrate specific antigens in cells and tissue based on the antigen antibody interaction and it exploit the specificity at light microscopic level[1]. Immunohistochemistry play an important tool in identification of new vessels.

Major components in a immunohistochemistry :

1. Primary antibody binds to specific antigen
2. The antibody-antigen complex is formed by incubation with a secondary, enzyme-conjugated, antibody
3. With presence of substrate and chromogen, the enzyme catalyzes to generate colored deposits at the sites of antibody-antigen binding.

Various stages of development of Immunohistochemistry include peroxidase – antiperoxidase method (1970), alkaline phosphatase labeling method(1971), avidin biotin method (1977) and two layer dextrin polymer technique(1993).

Steps of immunohistochemistry:

Antigen retrieval :

Antigen retrieval is done to unmask the antigen determinants of fixed tissue sections.This can be done by

1. Proteolytic enzyme digestion
2. Microwave antigen retrieval
3. Microwave and trypsin antigen retrieval
4. Pressure cooker antigen retrieval

Proteolytic enzyme digestion:

Enzymes like trypsin and proteinases are used to breakdown the formalin cross linkages and unmask the antigen determinants.But there is a disadvantage of antigen destruction and inadequate digestion.

Microwave antigen retrieval:

In this formalin fixed paraffin sections are boiled in various buffers for rapid and uniform heating. Currently this is the most common method used.

Pressure cooker antigen retrieval:

In this method also the tissue sections are boiled in buffers to unmask the antigens. This method is used to retrieve large number of slides.

Detection systems:

After adding specific antibodies to the antigens, the antigen antibody complex should be detected.This is done by direct and indirect methods.

Direct method:

The primary antibody is directly conjugated with flurochrome.Commonly used flurochromes are horse radish peroxidase and alkaline phosphatase.

Indirect method :

It is a two-step method**.**

First the labeled secondary antibody reacts with primary antibody which is bound to specific antigen. The use of peroxidase enzyme complex or avidin biotin complex further increases the sensitivity of immunohistochemical stains.

There are certain drawbacks are associated in evaluation of microvessel density in breast cancer,

According to Astekar et al., (2012), [134] differences between various studies could be due to different antibodies used like CD 31, CD 34 and factor VIII by authors to define endothelium and different methodologies used in assessment of various parameters**.**

Tae et al., in 2000, [135] stated lack of a standardized direct method to measure angiogenesis as a factor. Also none of the existing methods can differentiate between resting and active angiogenic vessels.

Astekar et al., [136] also stated that differences between immunohistochemical protocols, like selection of the paraffin block, level of section within the tissue block, that is, superficial or deep and variability in the selection of hot spot identification may contribute to the variation of the results**.**

Therefore, we needed new endothelial cell markers that would detect only active neoangiogenic vessels.

ROLE OF CD 34 IN DETECTION OF MICROVESSELS DENSITY:

CD34 molecule is a cluster of differentiation molecule present on certain cells within the human body.

Cd 34 also known as haematopoietic progenitor cell antigen 34. It is a glycosylated type I protein comprosed of 385 aminoacids and having a molecular weight of 41 kDa encoded on the gene 1q32.

There are two forms as result of alternative splicing.

Cd34 is expressed on haematopoietic stem cells of all lineages, as well as most primitive stem cells.

Cd 34 antigen also found in dermal dentrocytes, interstitial cells, endometrial stroma, endothelial cells of blood vessels, hepatic sinusoids, fibroblasts. It also expressed in hemangiomas, epitheloid hemangioendothelioma, Kaposi sarcoma, angiosarcomas.

Commercially available preparations are My 10,qb end/10, bi -3c5, 4H11,43A1 and TUK3 and few rabbit monoclonal antibodies EC373Y.

da Silva BB et al stated that the mean microvessel densities with anti-Factor VIII, anti-CD31 and anti-CD34 were 4.16, 4.09 and 6.59 respectively. The density and intensity of staining of anti-CD34 is also greater compared to anti-CD31 and anti-factor VIII-related antigen.[137]

DEVELOPMENT OF QUANTITATIVE ASSAYS FOR ANGIOGENESIS:

In the year 1980 Folkman and his colleagues successfully established the appropriate conditions to grow endothelial cells in culture medium, and used them to determine the functional steps in angiogenesis [138]. Thereafter a number of quantitative and semi quantitative assays have developed that involve sprouting angiogenesis in non tumourous cells [139], like

- corneal micropocket assay
- the subcutaneous `Matrigel plug' assay [139]

- assays involving growth of cut sections of blood vessels in three dimensional gels of an extracellular matrix**.**[140].

The introduction of these systems has led to the discovery of new proangiogenic and anti angiogenic factors

CLINICAL APPLICATION:

Though, microvessels density is used as prognostic factor in breast carcinoma, microvessel count has not been proved to be a acceptable parameter to estimate antiangiogenic treatment**.**

The assumptions that the degree of tumour angiogenic activity is proportional to the degree of microvessel count and that the quantification of microvessel density will gives the much needed information for antiangiogenic therapy, have led to two misconceptions :

1. The level of microvessels density in untreated tumour can asses the response to antiangiogenic treatment,
2. Microvessels density could evaluate the efficacy of the antiangiogenic drugs.

Microvessel density varies widely with tumor type. The growth of all tumor types depend on a therapeutically targetable angiogenic process.

According to Rajkumar SVet al, Munshi NC[141] et al,the growth of the tumour depends on the levels of oxygen exchange and nutrient supply,which indirectly reflects the intensity of tumour angiogenesis.

The metabolic demands of tumour cells vary with the tissue of origin and change with tumor progression. Thus, the number of tumor cells that can be supported by a vessel varies, influencing, in turn, the vascular density of the tumour.

Beecken WD, Fernandez A et al.[142] showed that tumour with both high and low level of microvessel density can respond to antiangiogenic treatment**.**

According to studies conducted by Folkman J, Hahnfeldt Pet al B[143], growth of all tumors depend on angiogenesis, hence tumour with low microvessel counts are not an appropriate criteria to omit patients from treatment with antiangiogenic agents.

A common misconception is that measurement of microvessel density made during antiangiogenic therapy may be used to evaluate the therapeutic response to antiangiogenic agents.

However, the efficiency of an antiangiogenic drug cannot be estimated by simply measuring the changes in microvessels count alone**.** This is because changes in microvessels density reflect the change in the ratio of the tumour vascularisation to its tumor-cell component.

Under antiangiogenic therapy, elimination of capillary occurs first, followed by elimination of tumour cells, and both make an impact on the measurement of microvessel density.

Thus, decrease in microvessel density while on treatment with an antiangiogenic drug implies that the drug is active, though microvessel density would not predict the tumor response to antiangiogenic therapy.

Currently, there is a wide range of investigations being carried out to find the basic biology of angiogenesis [144-147], to provide further insights into the effective use of antiangiogenic drugs [148] .Thus,antiangiogenic agents are classified in to different types based on the target [149], mode of action, or stage of cancer most appropriate to antiangiogenic agents in order to provide a standard guidelines for using combination therapy. [150-152], hence in order to resolve, supplementary assays may be needed other than microvessels density for evaluating the efficiency of antiangiogenic drugs.

Methodology

MATERIALS AND METHODS

This is a both retrospective and prospective study was conducted in the Department of Pathology, Tirunelveli Medical College for a period of about 24 months from june 2012 to june 2014. From 225cases of breast specimens 50 cases of breast specimens with known malignancy were randomly selected and examined, which included 30 cases of lymph node positive breast carcinoma specimens and 20 cases of lymphnode negative breast carcinoma specimens. This study was conducted after obtaining clearance from the Institutional Ethical Committee.

Immunohistochemistry was done on all the 50 cases inorder to findout microvessels in intratumoural and peritumoural area with cd 34 marker.

INCLUSION CRITERIA:

Cases of breast carcinoma were included.

IMMUNOHISTOCHEMICAL TECHNIQUE :

Immunostaining with the endothelial cell marker CD34 was performed following an avidin-biotin immunoperoxidase procedure.

Primary Antibody Kit:

Specificity	: Human endothelial cells-CD34 antigen
Clone	: QBEnd/10
Ig Class	: Ig G1

Antigen used for immunization : Detergent solubilized vesicular suspension prepared from a perfusate of human term placenta.

Hybridoma Partner : Mouse Myeloma.

Preparation : Lyophilised tissue culture supernatant containing 15 mmol sodium azide.

Effective on paraffin wax embedded tissue**.**

Secondary Antibody Kit :

Contains

1. 3% Hydrogen Peroxide,
2. Phosphate-buffered saline with stabilizers,
3. Polymer penetration enhancer -10% (v/v) animal serum in tris buffered saline (0.09%),
4. Anti-mouse/rabbit IgG-Poly-HRP,
5. DAB Chromogen 1.74% w/v 3,3'-diaminobenzidine, 0.02% Hematoxylin.

Reagents required but not supplied in secondary antibody Kit include Standard solvents used in immunohistochemistry, 50 mM tris buffered saline (TBS), Antigen retrieval solution, Enzyme retrieval solution, Antibody diluents, Primary Antibody, Mounting medium.

The primary monoclonal antibody was used at a dilution of 1:500 (DAKO Envision, Santa Cruz Biotechnology), previously slides were treated

by incubation in pressure cooker for 20 minutes at 1.5 atmospheres with citrate buffer pH 6.1. After washing the samples three times with Tris Buffered Saline (TBS), release reaction was made by adding DAB chromogen (3,3'diaminobenzidine) as a chromogen staining substrate for 5-10 minutes. Tissue sections were counterstained with hematoxylin.

Day 1 Procedure:

Sections are cut from paraffin embedded tissue blocks. Blocks are fixed in egg albumin coated slides**.**Slides are kept in hot air oven overnight at 60°C**.**

Day 2 Procedure:

- Make up citrate buffer by adding anhydrous citric acid 1.92 grams in 1000 ml of distilled water**.** adjust the ph to 6.2 with 1 N of sodium hydroxide.
- Then, make up TRIS saline buffer by TRIS 0.605 grams and 8 grams of NaCl in 1000ml of water, add 4 ml of 1 N Hcl**.**maintain the Ph for 7.6.
- Slides were deparaffinized with four washes of xylene. Slides are rehydrated by propanol**.**
- Slides rinsed in running tap water**.**
- Slides were kept in citrate buffer, in the pressure cooker for antigen retrieval for 20-25 minutes and then cooled for 15 minutes.

- Slides were treated with Hydrogen Peroxide for 5-10 minutes for elimination of endogenous peroxidase activity.
- Slides were rinsed in running tap water for 5-10 minutes**.**
- Slides were incubated with TRIS buffer saline for 5 minutes.
- 50 µl of NRS (1:10) was added to block non-specific binding sites and kept for 20 minutes.
- Primary antibody added with dilution and kept for 1 hour**.**
- Sections are soaked in Tris buffer for 10 minutes
- Suitable Polymer penetration enhancer was added and kept for 15 minutes, again wash with tbs for 3 min.
- Incubate with secondary antibody (igG-poly-HRP), 20 min. at room temperature.
- Three changes of tris buffer for five minutes.
- Stain with diaminobenzidin (DAB) solution, 5-10 min. at room temperature.
- Wash the slides with running tap water for 2-3 min.
- Counterstain with Mayer's hematoxylin, 3 dips.
- Again wash the slides with running tap water.
- Dehydrate with upgrading concentration of ethanol: 50%, 70%, 96%, absolute, 3 min. each.
- Clear with xylene.
- Mount with DPX and view.

MORPHOMETRIC ANALYSIS:

To quantify vessel neoformation we used hot spot method according to Weidner method.

After CD34 staining, each case was evaluated in order to select the area with the highest vascularization. For this purpose a scope image 9.0exe microimaging device was used.

Brown-stained cell groups clearly distinguishable from the background were counted as one vessel. All groups of cells positive for CD34 endothelial cell marker but without an evident lumen, were considered as undifferentiated vessels. Vascular hot spots were identified at a low optical power using a 4x and10x objective. Four equal areas of high vascularization were photographed with a 10x objective.

Measurements were performed using the Scope image 9.0 exe Image Analysis System**.**

Any artifacts occurring in the samples were removed manually.

Vessels were identified according to caliber into the following categories:

- Capillaries: Max.diameter- 8-15 μm
- Arterioles: Max.diameter- 15-50 μm
- Small Arteries: Max.diameter - 50-200 μm

was considered for area.

DIGITAL EVALUATION OF MVD :

The recorded slide images of each case were manually assessed using scope image 9.0 exe software and measurement scales were calibrated for 4x, 10x and 40x power**.** The average MVD, average lumen area (μm2), average vascular area (μm2), and median vessel wall thickness (μm) were measured. MVD was defined as number of vessels per square-millimeter. Vascular area was defined as the sum of the areas of all the endothelial cells of a vessel. Adjusted MVD as defined by Tanigawa was obtained using microvessel density × mean vessel perimeter (μm) / 40 (μm).

STASTICAL ANALYSIS:

The data were statiscally analysed using spss statistical software. Two talied student t test and one way ANNOVA test were used to asses the association between two quantitative variable. Kendall tau rank correlation coefficient test was used to measure association between two measured quantities. The p-value less than 0.05 were considered significant.

OBSERVATION

AND RESULTS

OBSERVATION AND RESULTS

This is a both retrospective and prospective study was conducted in the histopathology Laboratory of the Department of Pathology, Tirunelveli Medical College for a period of 24 months and the following observations were made.

TABLE 3- BREAST SPECIMENS VS OTHER SPECIMENS

Duration	Total Number Of Specimens	Number Of Breast Specimens	Percentage
June2012-December 2012	1870	48	2.566
January2013-December 2013	3518	122	3.467
January 2014-june 2014	1960	55	2.806
Total	7348	225	3.062

In the study period of 24 months duration from June 2012 to june 2014, total number of specimens we received are 7438. Out of these

specimens, the 225 specimens of breast cases giving a percentage of 3.062%.

48 cases of breast specimens were received of 1870 total specimens were studied during the first 6 months period in 2012 constituting 2.566%.

122 cases of breast specimens were received of 3518 total specimens were studied during the second 12 months period in 2013 constituting3.467%. The following 6 months study in 2014 showed 55 breast specimens cases out of 1960 of total specimens received which constituting2.806%, as shown in Table 3 and Chart 1.

CHART 1- BREAST SPECIMENS vs OTHER SPECIMENS

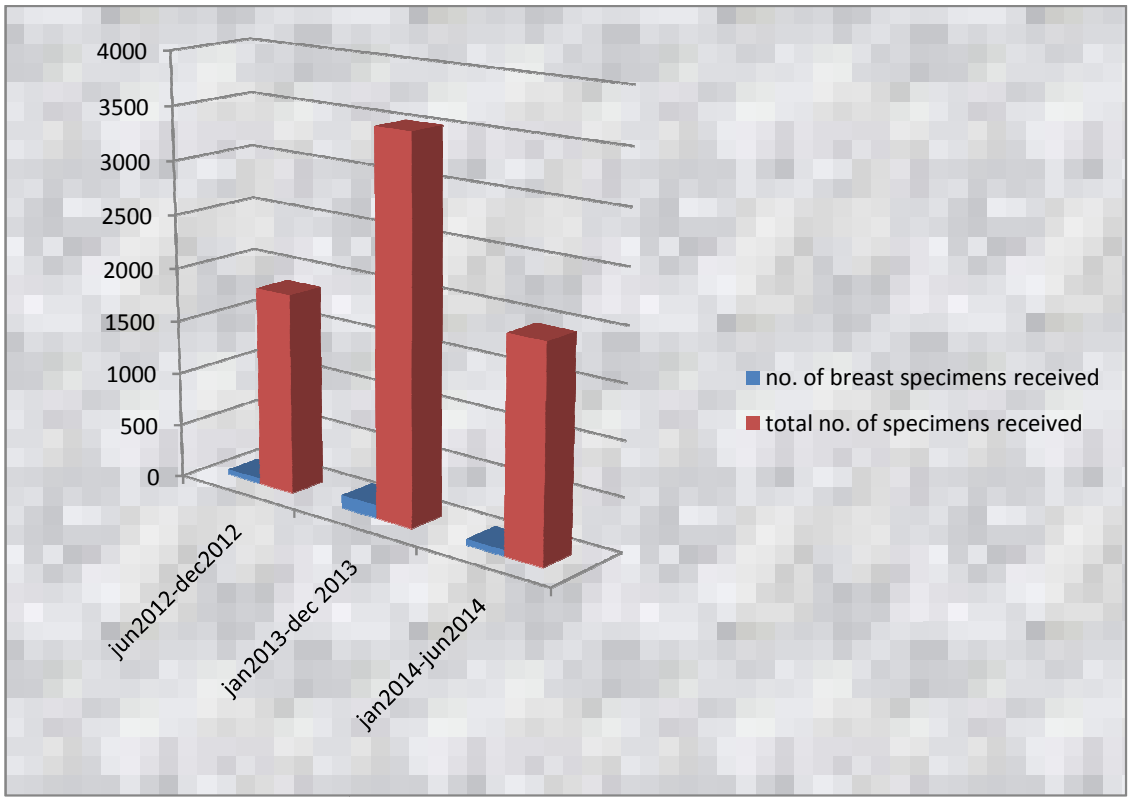

TABLE 4- AGE DISTRIBUTION OF BREAST CARCINOMAS

Age Groups	Number Of Breast Carcinomas	Percentage (%)
30-39 yrs	2	4%
40-49 yrs	17	34%
50-59 yrs	19	38%
60-69 yrs	9	18%
70-79 yrs	3	6%
	Mean age = 52.58	

By dividing the age group of patients into five categories, we observed that breast carcinomas were more common in the age group of 50 – 59 years with 19 cases constituting 38%, followed by the age group of 40 – 49 years with cases comprising 34 % and next to follow is the age group of 60 – 69 years with 9 cases constituting 18%. The youngest patient in our study is 35 years old and the oldest patient in our study is 75 years of age. We arrived at a mean age of 52.58 years for occurrence of breast carcinoma in our study. These findings are tabulated in Table 4 and Chart 2.

CHART 2- AGE DISTRIBUTION OF BREAST CARCINOMAS

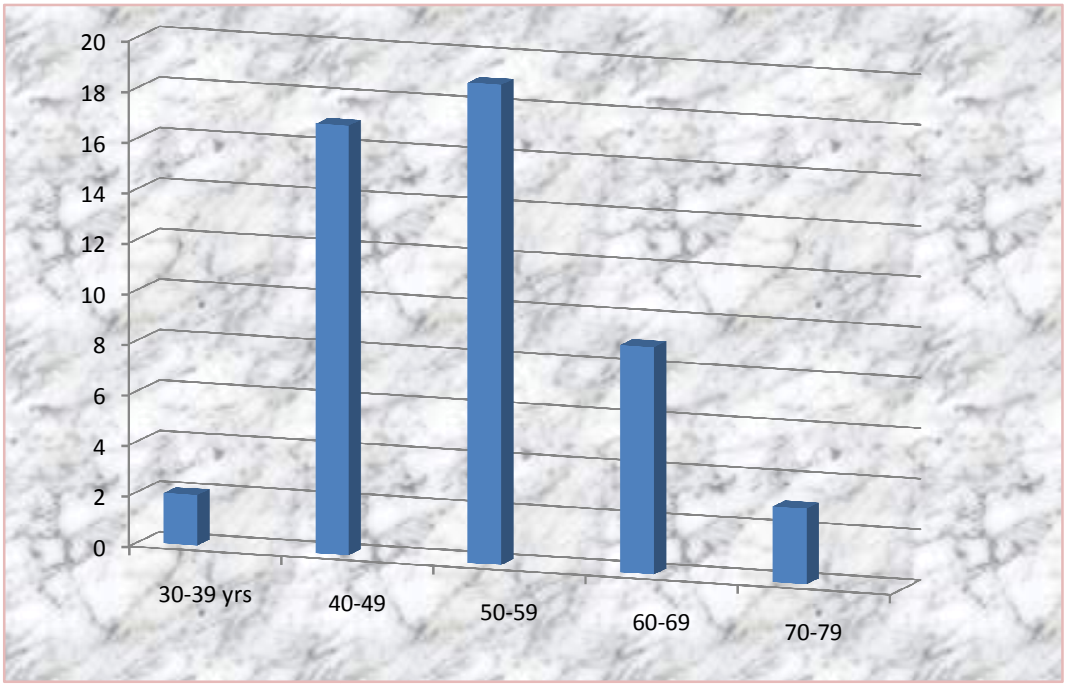

TABLE 5- HISTOLOGY GRADE DISTRIBUTION OF BREAST CARCINOMAS

Grade	Number of Cases	Percentage (%)
Grade I (3-5)	2	4%
Grade II (6-7) score	40	80%
Grade III (8-9) score	8	16%
Total	50	100%

All 50 cases of breast carcinomas were graded according to modified Blooms and Richardsons grading system into three grades. Grade I carcinoma constituted 2 cases with a percentage of 4%, grade II carcinoma constituted the majority with 40 cases constituting 80% and grade III carcinoma constituted 8 cases with a percentage of 16%. These observations are shown in Table5 and chart-3

CHART3-HISTOLOGY GRADE DISTRIBUTION OF BREAST CARCINOMAS

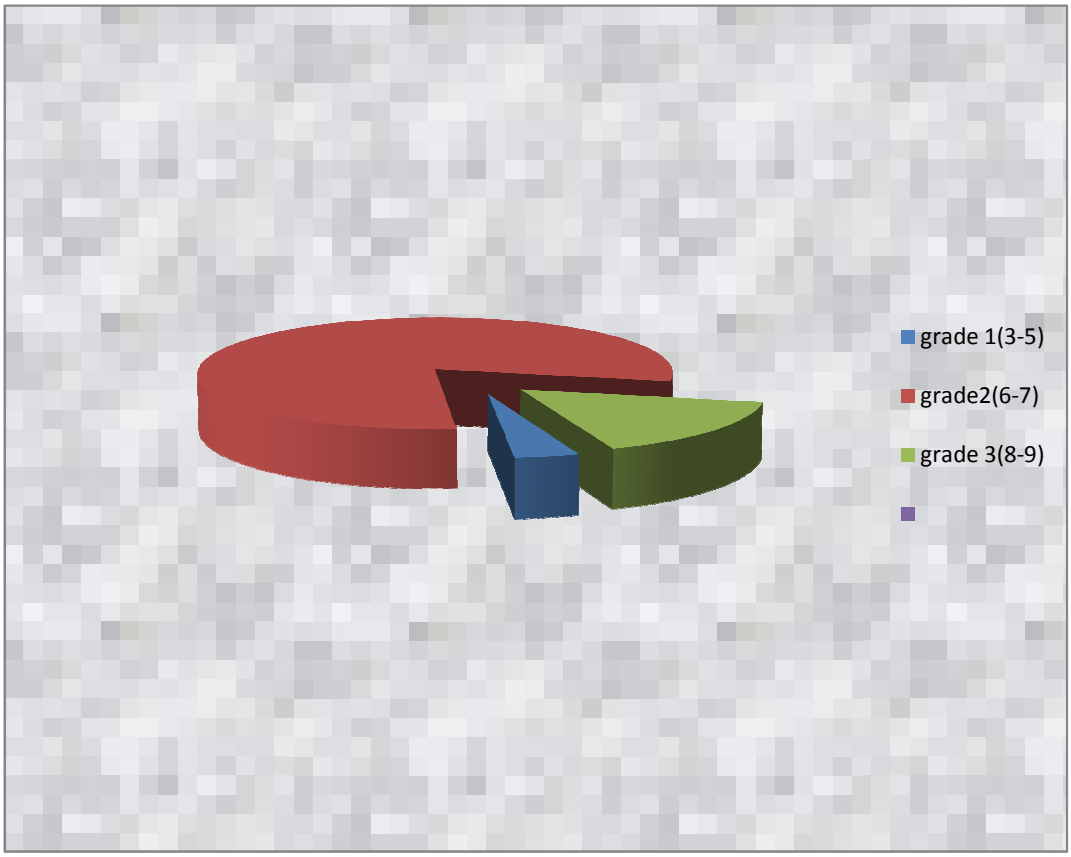

TABLE 6- HISTOLOGY GRADE vs TUMOUR SIZE

Size of tumour (cm)	Grade I (%)	Grade II (%)	Grade III (%)	Total	Percentage
<2 (T1)	1(50%)	1(2.5%)	0	2	4%
2– 5 (T2)	1 (50%)	26 (65%)	1(12.5%)	28	56%
>5 (T3)	0	13 (32.5%)	7 (87.5%)	20	40%
Total	2	40	8	50	100%

The association between histological grade and tumour size was made in the Table 6and Chart 5, which revealed most of the tumours (56%) to be in T2 (2 – 5cms) size. Among the Grade I tumours 50% (1 cases) were in T2 and 50% (1cases) were in T1. Among the Grade II tumours 65% (26 cases) were in T2 and 32.5% (13 cases) were in T3. Among the Grade III tumours 12.5% (1 cases) were in T2 and 87.5% (7case) was in T3 size. On applying the chi square test for statistical significance the P-value was **0.001** – sig. Hence the correlation between histological grade and tumour size was significant.

CHART 4- HISTOLOGY GRADE vs TUMOUR SIZE

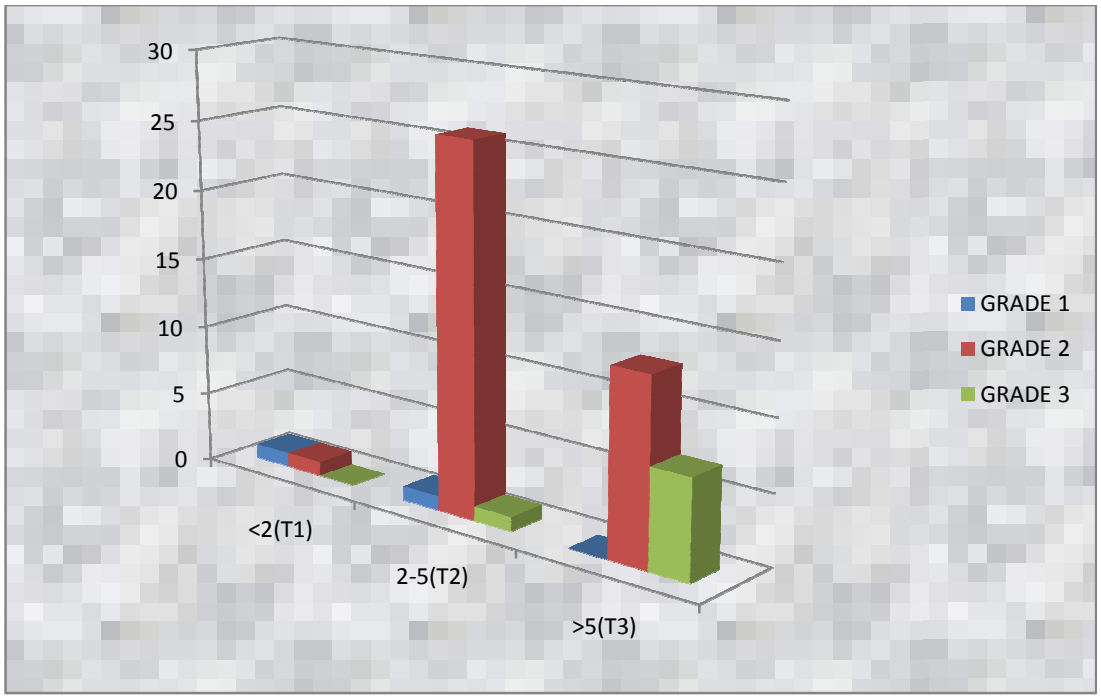

TABLE7- HISTOLOGY GRADE vs PATIENT AGE

Patient age	Grade I (%)	Grade II (%)	Grade III (%)	Total	Percentage
30-39 yrs	0	2	0	2	7.5%
40-49 yrs	2	14	1	17	42.5%
50-59 yrs	0	12	7	19	30%
60-69 yrs	0	9	0	9	15%
70-79 yrs	0	3	0	3	5%
Total	2	40	8	50	

The Table 7 and Chart 5 depicts that grade III tumours were more common in the age group of 50 to 59 years followed by the age group of 40 to 49 years constituting 16.7%. The age group of 40 to 49 years is the most common age group affected in both grade I (100%) and grade II (46.2%) tumours. Statistical calculation were analysed by chi-square method, there was no statistical correlation between patient age and tumour grade (P-value – **0.088411**> 0.05 – Not sig).

TABLE8- HISTOLOGY GRADE vs LYMPH NODE STATUS

Lymph node status	Grade I (%)	Grade II (%)	Grade III (%)	Total	Percentage
Positive	1(50%)	21(42.3%)	8(100%)	30	42.5%
Negative	1 (50%)	19 (57.7%)	0	20	57.5%
Total	2	40	8	50	100%

Among the grade I tumours 50% showed negative lymph node status and 50% showed positive lymph nodes. On the other hand grade III tumours showed 100% positive and 0% negative lymph node status. Grade II tumours showed equivocal findings with 42.3% showing lymph node positivity and

57.7% showing lymph node negativity. These findings are depicted in Table 8 and Chart 6. Chi-square method was employed,it was found that there is no statistical correlation between grade of the tumour and lymph node status (P-value – **0.041707**>0.005).

CHART 5- HISTOLOGY GRADE vs PATIENT AGE

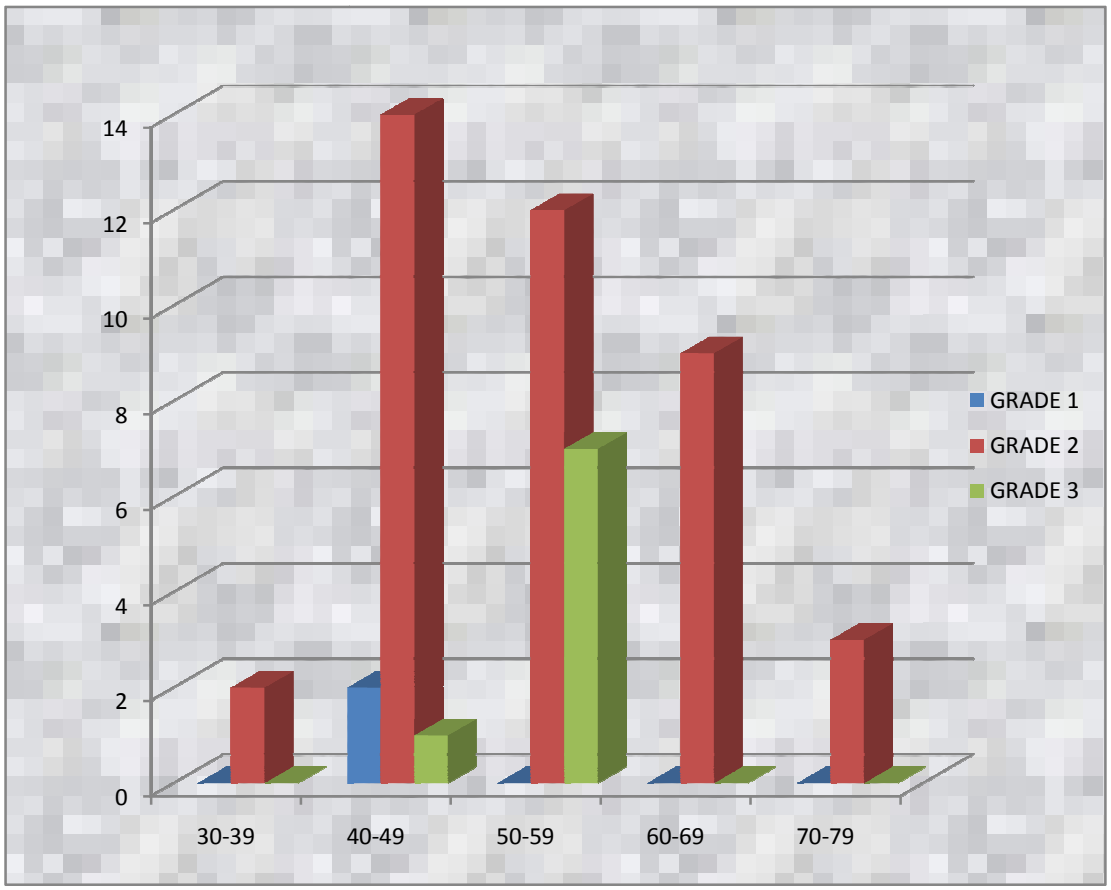

CHART 6-HISTOLOGY GRADE vs LYMPH NODE STATUS

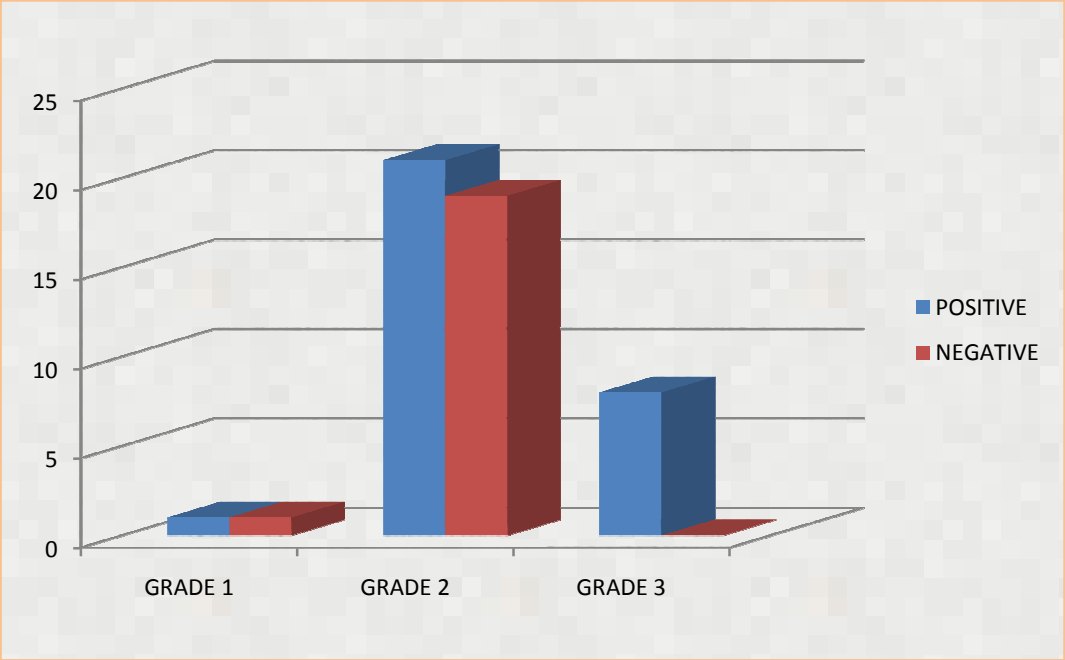

TABLE -9 MICROVESSEL DENSITY GRADE DISTRIBUTION OF BREAST CARCINOMAS.

Microvessel Grade	No. Of Breast Carcinomas	Percentage
Grade –I(0-28)	3	6%
Grade –II(29-56)	17	34%
Grade –III(57-84)	10	20%
Grade – IV(>84)	20	40%
TOTAL	50	100%

All 50 cases of breast carcinomas microvessels were counted and divided into four grades. Grade I microvessel density constituted 3 cases with a percentage of 6%, grade IV carcinoma microvessel density constituted the majority with 20 cases constituting 40% and grade II carcinoma constituted 17 cases with a percentage of 34%. These observations are shown in Table 9 and chart -7

CHART 7- DISTRIBUTION OF MICROVESSELS COUNT IN BREAST CARCINOMAS:

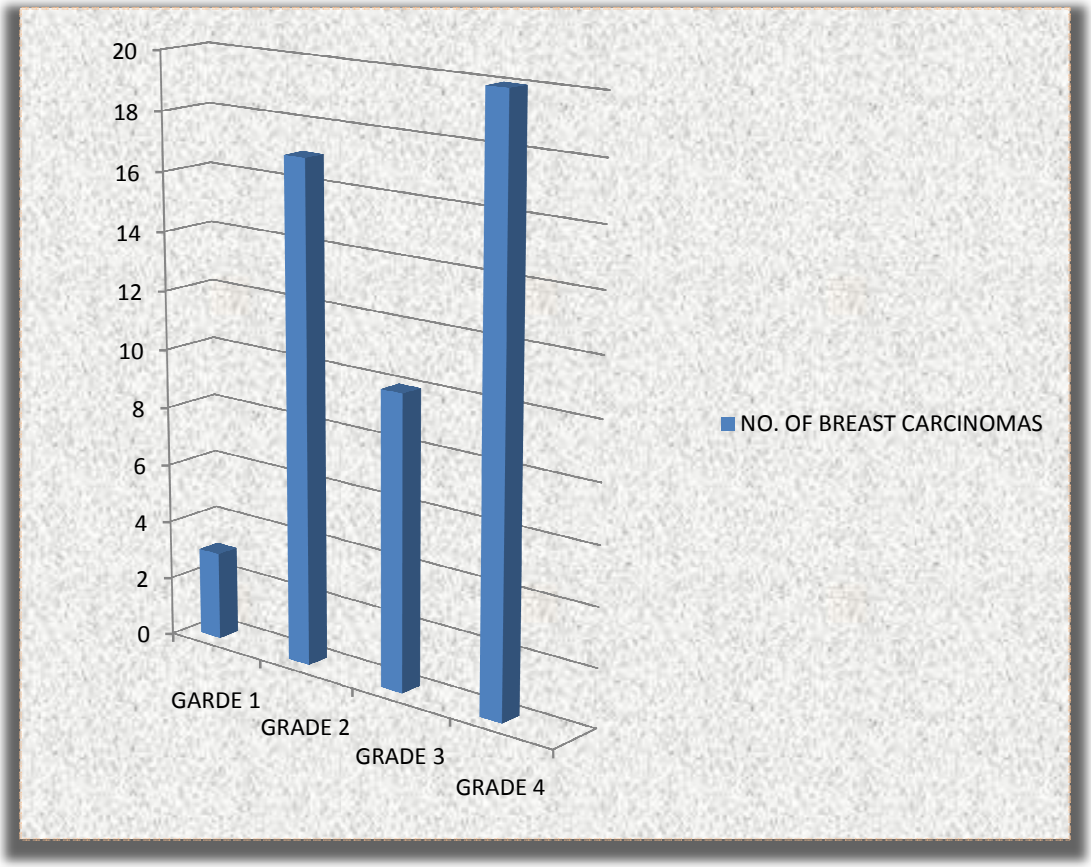

TABLE- 10- MICROVESSEL COUNT –INTRATUMORAL VS PERITUMOURAL.

	MICROVESSELS COUNT			
	Mean	Standard Deviation	Range	P-Value
INTRATUMOURAL MICROVESSELS.	70.2	28.17	22-134	p-0.001
PERITUMOURAL MICROVESSELS.	91.58	28.09	45-174	

The mean microvessels density within the tumour has lesser number of microvessels as compared to Peritumoural area. The mean microvessels density in intratumoral area is 70.2 with standard deviation of 28.17 where as the mean microvessels density in peritumoural area is 91.58 with standard deviation of 28.09. p – value for both these group were analysed by paired student t- test, it was found to be **<0.001** (statistically significant).

TABLE-11 : PERITUMOURAL MICROVESSEL COUNT VS TUMOUR SIZE

	PERITUMOURAL MICROVESSELS COUNT			
Tumour Size	No. Of Breast Carcinomas	Mean Value	Standard Deviation	P- Value
<2 cm	2	61.5	7.778	p-0.240
2-5 cm	28	87.607	24.914	
>5 cm	20	93.6	28.059	

There was a poor correlation between the MVD values and the tumour size. The mean values of peritumoural microvessels counts increased gradually as the size of the malignant neoplasm increased, with T1 lesions showing a microvessels count of 61.5, T2 lesions showing a microvessels count of 87.607 and T3 lesions showing the highest mean peritumoural microvessels count of 93.6. But, the correlation between peritumoural microvessels count and the size of the breast neoplasm was not stastically

significant with (P-value – **0.240** >0.05). These observations were analysed by one way- ANNOVA test and it is depicted in table-11.

TABLE -12:PERITUMOURAL MICROVESSELS COUNT VS TUMOUR GRADE

Tumour Grade	PERITUMOURAL MICROVESSELS COUNT			
	No. Of Breast Carcinomas	Mean	Standard Deviation	P- Value
Grade-1	2	61.5	7.7	p-0.126
Grade-2	40	87.8	27.36	
Grade-3	8	101.625	15.436	

The microvessels counting was done on 50 cases which revealed mean peritumoural mirovessels count of 61.5 in grade 1 tumours, while mean peritumoural microvessels count was higher in grade 3 with a score of 101.625+/-15.436. The mean peritumoural microvessels count showed higher values in grade 3 tumours compared to grade1 breast carcinomas. Peritumoral microvessels count and grading of tumours were analysed by one way-ANOVA test,it was not statistically significant(P value – **0.126)**,

however it has shown a positive correlation between tumour grade and microvessels density, analysed by kendals rank correlation coefficient method. These findings are tabulated in Table 12**.**

TABLE-13:PERITUMOURAL MICROVESSELS COUNT VS LYMPH NODE STATUS

	PERITUMOURAL MICROVESSELS COUNT			
Lymph Node Status	No. Of Breast Carcinomas	Mean Value	Standard Deviation	P- Value
Positive	30	103.2	21.063	p-0.001
Negative	20	69.15	17.31	

The patients with positive lymph node status shows higher Peritumoural Microvesssels count compared to patients with negative lymphnode, the mean Peritumoural microvessels count for node positive status is 103.2 where as 69.15 in node negative status,two groups were

compared by paired student-t test, the results were found to be stastically significant with p- value **-0.00001**(significant).Peritumoral microvessel density values with regard to lymph node status are shown in Table 13

TABLE 14: PERITUMOURAL MICROVESSELS COUNT VS AGE

	PERITUMOURAL MICROVESSELS COUNT			
Age	No. Of breast carcinomas	Mean value	Standard deviation	P- Value
30-39	2	72.5	7.77	p-0.884
40-49	17	92.118	31.07	
50-59	19	86.7	23.036	
60-69	9	91.8	27.2	
70-79	3	90.3	26.57	

The Table 14 depicts that mean value of peritumoural microvessels count were92.118 in the age group of 40 to 49 years followed by the age group of 60 to 69 years constituting 91.8**.** The age group 30 to 39 which have the least mean peritumoural microvessels count of 72.5**.** There was no statistical significance between patient age and peritumoural microvessels count **p-0.884.**

TABLE-15 :INTRATUMOURAL MICROVESSEL COUNT VS TUMOUR SIZE

	INTRATUMOURAL MICROVESSELS COUNT			
Tumour Size	No. Of Breast Carcinomas	Mean Value	Standard Deviation	P- Value
<2 cm	2	33	15.556	p-0.156
2-5 cm	28	69.214(25-12O)	27.03	
>5 cm	20	76.500(27-134)	29.752	

There was a poor correlation between the MVD values and the tumour size. The mean values of intratumoural microvessels counts increased gradually as the size of the malignant neoplasm increased, with T1 lesions showing a microvessels count of 33, T2 lesions showing a microvessels count of 69.214 and T3 lesions showing the highest mean microvessels count of 76.500. But, the correlation between microvessels count and the size of

the breast neoplasm was not stastically significant with (P-value **– 0. 156** >0.05). These observations were depicted in table 15

TABLE -16: INTRATUMOURAL MICROVESSELS COUNT VS TUMOUR GRADE

Tumour Grade	INTRATUMOURAL MICROVESSEL COUNT			
	No. Of Breast Carcinomas	Mean	Standard Deviation	P- Value
Grade-1	2	38(35-45)	7.07	p- 0.005
Grade-2	40	60.75(22-108)	17.02	
Grade-3	8	93.37(56-134)	25.41	

The intratumoural microvessels counting was done on 50 cases which revealed mean mirovessels count of 38+/-7.07 in grade 1 tumours, while mean microvessels count was higher in grade 3 with a score of 93.37+/-25.41. The mean microvessels count showed higher values in grade 3 tumours compared to grade1breast carcinomas. Microvessels count and grading of tumours were analysed by one way-ANOVA test,it has shown a

stastically significant values, higher values in grade 3 tumours compared to the grade -1 tumours (P value – **0.005**) and the values of intratumoural microvessels density correlated well with tumour grade (kendals rank correlation co-efficient). These findings are tabulated in Table 16

TABLE 17: INTRATUMOURAL MICROVESSELS COUNT VS LYMPH NODE STATUS

	INTRATUMOURAL MICROVESSELS COUNT			
Lymph Node Status	No. Of Breast Carcinomas	Mean Value	Standard Deviation	P- Value
Positive	30	85.63(25-134)	24.318	p-0.00001
Negative	20	47.60(22-79)	15.44	

The patients with positive lymph node status shows higher intra tumoural microvesssels count compared to patients with negative lymphnode, the mean microvessels count for node positive status is 85.63 where as 47.60 in node negative status,two groups were compared by one

way ANNOVA test, the results were found to be stastically significant with p- value -**0.00001**(significant), are shown in Table 13

TABLE-18 - INTRATUMOURAL MICROVESSELS COUNT VS AGE

	INTRATUMOURAL MICROVESSELS COUNT			
Age	No. Of Breast Carcinomas	Mean Value	Standard Deviation	P- Value
30-39	2	49.5	6.364	p-0.825
40-49	17	73.25(22-134)	33.007	
50-59	19	67.947(25-112)	26.328	
60-69	9	71.556(25-119)	32.639	
70-79	3	68.33(43-96)	26.577	

The Table 18 depicts that mean value of intratumoural microvessels count were 73.25 in the age group of 40 to 49 years followed by the age group of 60 to 69 years constituting 71.556**.** The age group 30 to 39 which have the least mean microvessels count of 49.5**.** ONE WAY –ANNOVA showed that here was no statistical correlation between patient age and microvessels count p value-**0.825..**

Discussion

DISCUSSION

Breast cancer is the leading cause of cancer deaths among women. Most of the studies suggested that tumor growth and metastasis in breast cancer depends on angiogenesis.

The American College of Pathologists suggested that an improved study on quantification of tumor angiogenesis is needed to demonstrate its prognostic value in breast carcinoma.

Angiogenesis is now growing as an important prognostic indicator in tumor progression. It has been stated that the number of micro vessels within a tumor gives an estimate of the angiogenic potential of tumor cells, which in turn yield the probability of tumor growth, invasion, and metastasis.

It has been confirmed in many solid tumor types such as melanoma, prostate, and breast cancer. Tumor angiogenesis is usually quantified by counting the number of micro vessels in tumour sections by immunohistochemical methods using endothelial cell markers, such as Factor VIII, CD31, CD34**.**

Takao Kato et al found that in certain Japanese breast cancer patients, especially in node-negative patients, there was a significant independent prognostic factor associated with their long term survival[153,161]**.**

However, results of various studies on the prognostic value of MVD have not been similar, possibly because of factors such as methodological

variation, a lack of correct patient follow-up data in retrospective studies, bias in selecting areas of tumors for study.

Degree of vascularisation in different cancers and angiogenesis was not uniform within the same tumor. So, the areas with highest micrvessels density called hotspots were counted.

In a study done by H P Dhakal et al[162] and Fox B et al[163], the Chalkley method appears to be the better method in estimating the prognostic impact of vascularity in invasive breast carcinomas

It has been stressed that the prognostic significance of tumor microvessels density must be assesed in a prospective way with a standardized methodology.

As proposed by Weidner et al[156], we counted the microvessels density by finding the vascular hotspots at 40x and point count at 100x magnification. we had taken one monoclonal antibody to CD34 and counted four fields/0.25 mm2 and the sum of all the four areas were calculated, this would give exact measurement of microvessels in the hot spot areas, as stated by L Martin et al[152a]. Earlier studies only evaluated in 200x per one microscopic field area for micro vessel density and this was analysed by statistical methods.

AS L Martin[152a] et al concluded that the microvessel density measured in histological sections were representative of whole tumour vascularity, based on this study we had taken the representative sections from the tumor.

Microscopic analysis for tumor areas with greater microvessel density establish a better correlation between intra tumoral vascularisation and tumor's angiogenic potential, hence we also followed this method.

Following the original study done by Weidner et al. in 1991, other researchers have also revealed the importance of angiogenesis in prognosis of breast cancer**.** In our study, we compared tumour angiogenesis measured by microvessels count with various other clinico-pathological factors like patient age, tumour size, histological grade, lymph node status, prognosis**.**

We also try to find any association between micro vascular count and other prognostic factors like Tumor size, axillary lymph node status, Tumor type, histologic grade, vascular invasion.

In this study the mean and median of microvessels density was 70.54 with a standard deviation of 28 (average Range, 25 to 134) for an area of 1 mm^2**.** The mean and median microvessel counts from the Weidner et al study was 60 and 56 (range, 8 to 167), respectively, for a counting area 0.74-mm^2.Whereas Van Hoef et al[157] got higher ranges of 67 and 101 respectively, for an area of 0.476- mm2.

Unlike the study by Horak et al[154], our study could not find any significant relationship between micro vascular density and size of the tumor with P value of 0.156**.** However, A borderline significant correlation (P=0.005) between Micro vascular density and high-grade tumours was

found by this study similar to that found by Weidner et al, horak et al and Bosari et al[154,159,160].

In our study we found a statistically significant association between the microvessels density and the lymph node status, similar to the studies done by Horak et al, and Weidner et al and Bosari et al who found a significant relationship between Microvessels count and lymph nodes status.

Goulding et al[153], could not find any relationship between the two parameters. As a prognostic indicator for breast carcinoma, Bosari et al, Horak et al and Weidner et al found a significant difference in overall survival rates between cases with low and high microvessel density.

However, the association between Micro vascular density and prognosis was not found by goulding et al, Van Hoef et al, Costello et al. In this study we found the microvessel density was increasing with age but this was not significant (P value =0.9)**.**Maxine Orre et al[155] also observed the same results..

From the above discussion it can be inferred that there are many discrepancies between results obtained by different pathology laboratories, and a greater number of these can be ascribed to the variations in methodology.

While counting micro vessels, the choice of antibody for immunohistochemical staining is important. The selection of antibody to

detect endothelial cells is a midway between the sensitivity and specificity of the available markers.

A number of studies have been carried out on different endothelial markers and none of them have arrived to any universal agreement till now**.** This is because of the various factors viz tissue-processing regime, choice of fixative, antigen-retrieval system and visualisation method affecting the sensitivity and specificity of all endothelial markers used**.**

Majority of the retrospective studies on angiogenesis research in breast carcinoma showed that angiogenesis was a crucial new prognostic factor in early-stage breast cancer. This should be further studied in prospective controlled clinical trials to demonstrate if any adjuvant treatment can improve the prognosis of those at high risk**.**

The vascular endothelial marker CD34 used in our study to calculate the microvascular density which stained the micro vessels greater and more intensely as shown by L martin et al in 1997[152a] and Da silva BB et al in 2009.

Instead of CD 34, the Van Hoef group used antiCD-31 to highlight the microvessels. Hence, the microvessel densities obtained by them are in a greater range than what would be expected.

Horak et al also[154] revealed anti-CD31 to be the most sensitive endothelial marker for highlighting intratumor microvessels. These discrepancies are due to the methodological problems.

The design and size of the study also affect the results obtained. Most studies of tumour microvessels density in breast carcinoma have used less than the 50 patients recommended for analysing its usefulness as a prognostic and predictive factors. In our study the number of cases taken was fifty, hence we met the required sample size.

In the present scenario, microvessel density as a prognostic marker for breast cancer is not reliable. Intratumoral new vessel growth appears to be a necessary event but not the only prognostic factor for tumour metastasis. However, Microvessel count serves as a good prognostic marker to identify patients at high risk.

Though many contradictions and inconsistencies have been interpreted in the studies carried out, the majority of them showed that high microvessels count correlated well with prognosis, lymph node status and histological grade. These differences are due to the variations in the investigation methods employed, variability in immune staining, the process of selection and counting.

In the future, antibodies specific to proliferating endothelium, with the development of automated image analysis, can improve the accuracy and value of measuring angiogenesis-induced microvessel density.

Summary

SUMMARY

Angiogenesis is now growing as an important prognostic factor in tumor progression. It has been stated that the number of micro vessels within a tumor gives an estimate of the angiogenic potential of tumor cells, which in turn yield the probability of tumor growth, invasion, and metastasis.

In our study, we observed statistically significant association between the microvessels density and the lymph node status. the mean microvessels count for node positive status is 85.63 where as 47.60 in node negative status.

Mean microvessels density was found to be more superior in peritumoural area as compared to intratumoural area**.**we also observed positive correlation in microvessel density between peritumoral and intra tumoural region.

There was a poor correlation between the MVD values and the tumour size. And also there was no statistical correlation between patient age and microvessels count p-0.865. Histological Grade of the tumour has shown a positive correlation with intratumoural microvessels density, although they are not statistically significant between their groups.

However, results of various studies on the prognostic value of MVD have not been similar, possibly because of factors such as methodological variation, a lack of correct patient follow-up data in retrospective studies, bias in selecting areas of tumors for study.

Most of the studies stated that there is a lack of a standardized direct method to measure angiogenesis as a factor.

The differences between various studies could be due to different antibodies used like CD 31, CD 34 and factor VIII by authors to define endothelium and different methodologies used in assessment of various parameters**.**

Thus, the study of microvessels density gives insight to the tumour behaviour related to angiogenesis. Hence, new endothelial cell markers that would detect only active neoangiogenic vessels and a standardised methodology is needed to evaluate the microvessels density in breast carcinoma in future.

Conclusion

CONCLUSION

Assessment and evaluation of the microvessel density and their parameters in the present study on breast specimens has enlightened us to draw the following conclusions-

- All the vascular parameters showed consistently higher values for high grade compared to the low grade tumour of the breast. Mean microvessels density was found to be more superior in peritumoural area as compared to intratumoural area**.**
- The patients with positive lymph node status shows higher microvesssels count compared to patients with negative lymphnode, the mean microvessels count for node positive status is significantly higher than compared with node negative patients.

Thus, the study shows a positive correlation between the tumour angiogenesis and metastasis.

In the future, Antibodies specific to proliferating endothelium, together with the development of automated image analysis, may improve the accuracy and value of measuring angiogenesis-induced microvessel density.

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Appendix

APPENDIX I

QUANTIOCATION MICROVESSEL DENSITY IN BREAST CARCINOMAS BASED ON IMMUNOHISTOCHEMISTRY.

PROFORMA

PATH NO:_____. IP/OP NO:_____

PATIENT NAME: _____

AGE : _____ SEX :M/F____ UNIT /WARD:_____

ADDRESS:_____

_____

_____

CLINICAL

DIAGNOSIS:_____

1. PRESENTING COMPLAINTS:_____
2. PERSONAL HISTORY : _____
3. FAMILY HISTORY :_____
4. GENERAL EXAMINATION:_____ _____
5. LOCAL EXAMINATION :_____
6. HPE DIAGNOSIS :_____
7. INTRATUMOURAL MICRO VESSEL COUNT :_____.
8. PERITUMOURAL MICRO VESSEL COUNT :_____.
9. MICROVESSEL GRADE :_____.

Master Chart

S.No	I.P. No	PATH NO.	AGE	SEX	SIZE	BLOOM AND RICHARDSON S GRADE	LN STATUS	PERI TUMOURAL MVD - CD-34					INTRATUMOURAL MVD-CD-34					MVD GRADE	HITSTOPATH DIAGNOSIS
								MICRO VESSELS COUNT /0.25 mm2				sum mvd/ mm2	MICROVESSELS COUNT/0.25mm2				sum mvd/ mm2		
1	34065	1475/12	55	f	8	2	positive	27	31	19	26	103	15	26	34	10	85	4	invasive ductal carcinoma
2	37163	1598/12	52	f	4	2	negative	12	17	15	19	63	6	16	11	9	42	2	invasive ductal carcinoma
3	26969	1617/12	50	f	7	3	positive	31	22	41	23	117	34	15	18	41	108	4	invasive ductal carcinoma
4	37791	1695/12	40	f	5	2	positive	45	38	32	16	131	42	23	12	43	120	4	invasive ductal carcinoma
5	39701	1745/12	62	f	7	2	positive	24	41	22	17	104	7	35	23	25	90	4	invasive ductal carcinoma
6	42179	1900/12	40	f	9	2	positive	19	31	45	52	147	26	28	34	46	134	4	invasive ductal carcinoma
7	42291	1925/12	48	f	5	2	negative	12	17	21	24	74	11	26	12	6	55	2	invasive ductal carcinoma
8	43381	1973/12	49	f	6	2	positive	34	31	29	21	115	26	31	18	22	97	4	invasive ductal carcinoma
9	43227	1990/12	60	f	5	2	positive	41	29	33	21	124	29	45	21	24	119	4	invasive ductal carcinoma
10	63309	2095/12	41	f	6	3	positive	31	19	21	28	99	11	35	19	26	91	4	invasive ductal carcinoma
11	45533	2137/12	48	f	2.5	2	negative	9	11	23	14	57	9	11	7	5	32	2	invasive ductal carcinoma
12	5132	231/13	45	f	6	2	positive	25	31	27	20	103	39	17	18	12	86	4	invasive ductal carcinoma
13	9014	453/13	52	f	4	3	positive	32	21	49	15	117	30	36	16	24	106	4	invasive ductal carcinoma
14	11486	460/13	55	f	3	2	negative	34	13	21	31	99	24	19	14	18	75	3	invasive ductal carcinoma
15	18881	795/13	35	f	2	1	negative	19	21	16	11	67	5	9	16	15	45	2	invasive ductal carcinoma
16	23837	1068/13	50	f	6	2	negative	12	9	14	10	45	3	4	11	9	27	1	invasive ductal carcinoma
17	24145	1084/13	55	f	3.5	2	positive	13	14	10	9	46	6	10	3	6	25	1	invasive ductal carcinoma
18	25490	1128/13	45	f	3	2	negative	31	25	19	13	88	17	12	11	27	67	3	invasive ductal carcinoma
19	28188	1228/13	65	f	3	2	negative	33	16	15	23	87	22	12	14	14	62	3	invasive ductal carcinoma
20	29662	1306/13	59	f	6	3	positive	24	13	21	28	88	22	12	24	18	78	3	invasive ductal carcinoma
21	50643	2339/13	45	f	6	2	positive	23	27	49	29	128	18	45	27	19	109	4	invasive ductal carcinoma
22	50441	2371/13	60	f	3.5	2	positive	19	21	18	9	67	7	9	11	18	45	2	invasive ductal carcinoma
23	53794	2485/13	45	f	5.5	1	negative	12	17	14	13	56	6	9	6	14	35	2	invasive ductal carcinoma
24	55077	2614/13	37	f	4	2	positive	29	18	12	19	78	20	17	11	6	54	2	invasive ductal carcinoma
25	54768	2658/13	45	f	3	2	negatiive	8	18	12	16	54	10	8	14	11	43	2	invasive ductal carcinoma
26	56887	2675/13	64	f	4.5	3	positive	31	29	28	25	113	21	14	25	36	96	4	invasive ductal carcinoma
27	55233	2736/13	61	f	3	2	positive	29	26	27	27	109	30	11	32	14	87	4	invasive ductal carcinoma
28	57147	2750/13	61	f	6	3	positive	32	26	19	36	113	23	23	25	23	94	4	invasive ductal carcinoma
29	58523	2778/13	48	f	4	2	positive	28	27	21	22	98	27	16	20	14	77	3	invasive ductal carcinoma
30	56570	2803/13	42	f	3	2	negative	19	8	11	16	54	5	8	14	11	38	2	invasive ductal carcinoma
31	59443	2806/13	46	f	6	2	negative	23	25	19	22	89	10	8	12	26	56	3	invasive ductal carcinoma
32	59527	2807/13	50	f	8	2	positive	31	25	29	24	109	22	30	20	16	88	4	invasive ductal carcinoma
33	59628	2827/13	56	f	6	2	positive	35	23	27	34	119	30	24	42	16	112	4	invasive ductal carcinoma
34	61016	2889/13	55	f	2.5	2	negative	29	25	19	25	98	17	19	14	29	79	3	invasive ductal carcinoma
35	61040	2929/13	70	f	9	2	negative	21	19	11	14	65	11	10	8	14	43	2	invasive ductal carcinoma
36	63509	2993/13	55	f	14	3	positive	19	18	16	25	78	9	16	22	9	56	3	invasive ductal carcinoma
37	64465	3089/13	55	f	5	2	negative	19	23	31	8	81	15	16	9	19	59	3	invasive ductal carcinoma
38	70236	3405/13	85	f	4	2	positive	34	29	41	14	118	19	21	35	21	96	4	invasive ductal carcinoma
39	181	93/14	47	f	4	2	positive	27	31	39	21	118	25	40	9	21	95	4	invasive ductal carcinoma
40	1348	114/14	47	f	5	2	positive	25	27	22	35	109	31	26	17	13	87	4	invasive ductal carcinoma
41	3978	227/14	72	f	6	3	positive	19	16	18	25	88	22	10	9	16	69	3	invasive ductal carcinoma
42	5313	310/14	50	f	4	2	positive	21	19	23	24	87	10	23	21	9	63	3	invasive ductal carcinoma
43	4954	324/14	50	f	3	2	negative	22	9	18	18	67	15	11	11	7	44	2	invasive ductal carcinoma

S.No	I.P. No	PATH NO.	AGE	SEX	SIZE	BLOOM AND RICHARDSON S GRADE	LN STATUS	PERI TUMOURAL MVD - CD-34					INTRATUMOURAL MVD-CD-34					MVD GRADE	HITSTOPATH DIAGNOSIS
								MICRO VESSELS COUNT /0.25 mm2				sum mvd/ mm2	MICROVESSELS COUNT/0.25mm2				sum mvd/ mm2		
44	6864	418/14	45	f	4	2	negative	11	9	10	16	46	4	3	6	9	22	1	invasive ductal carcinoma
45	3586	466/14	62	f	2	2	negative	19	15	8	14	56	9	13	6	6	34	2	invasive ductal carcinoma
46	8315	470/14	55	f	5	2	positive	26	29	19	15	89	16	14	19	12	61	3	invasive ductal carcinoma
47	8924	492/14	50	f	6	2	negative	13	21	11	16	61	7	18	9	5	39	2	invasive ductal carcinoma
48	6855	521/14	54	f	5	2	positive	19	24	11	21	75	10	13	14	17	54	2	invasive ductal carcinoma
49	10175	556/14	56	f	4	2	positive	31	21	29	25	106	13	26	31	16	86	4	invasive ductal carcinoma
50	11156	601/14	65	f	7	2	negative	18	9	11	6	45	13	9	13	7	42	2	invasive ductal carcinoma

KEY TO MASTER CHART

LN STATUS	-	LYMPH NODE STATUS
MVD	-	MICROVESSEL DENSITY